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The role of Xist RNA in the maintenance of X chromosome inactivation

by

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Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology
at the
Massachusetts Institute of Technology
September 2001

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To David and Christopher
ACKNOWLEDGEMENTS

During my six years in graduate school, I received help, guidance and encouragement from many people. This is my place to thank them for everything, even though probably not one of them is going to read this page.

My thesis supervisor, Rudolf, was kind enough to take me into his lab, and to wait patiently for three months before I got my first ligation reaction to work. Eventually, I became more efficient, and I hope I did not disappoint him at the end. In the laboratory, I would have been lost without the expert help of our mouse specialists, Jessie Dausmann and Ruthie Flannery. I will also be eternally grateful to Jan Loring for her help with the maintenance of the mouse colony. Members of the X-inactivation group taught me many of the techniques I needed. Barbara Panning’s expertise in FISH and immunofluorescence and York Marahrens’ knowledge of homologous targeting were especially helpful. Caroline Beard and Brian Bates were instrumental in troubleshooting the Cre-loxP technology and made it easier for the rest of us to generate conditional mutations. Laurie Jackson-Grusby, besides being my collaborator on the project described in Chapter 4, lent me her old laptop computer that allowed me to write most of my thesis at home, away from the distractions of lab and close to my baby. I would also like to thank the younger generation of the X-inactivation group, especially Anton Wutz, for being a constant source of amusement in my bay. Anton, Sandra Luikenhuis and Ted Rasmussen were sources of many helpful ideas, comments and suggestions.

I would also like to thank my thesis committee, David Housman and Terry Orr-Weaver, for their advice and guidance throughout my graduate carrier. They helped me not only with my research project, but also with the what-to-do-with-my-life decision. I could not have asked for kinder and more caring committee members.

Finally, my graduate school experience would not have been the same without the many happy evenings we spent drinking beer and forgetting about science. Dale, Dave, Detlev, François, Matt, Maribel, and Britt, were good company for doing just that. Later,
in my more sober years, I switched to coffee drinking with Anton, Konrad, Joost, Ted, Sandra, and Suzanne. Thank you all, and I hope to see you some time soon for another pint/cup!
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Submitted to the Department of Biology on June 21, 2001 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

The role of Xist RNA in silencing the inactive X of female somatic cells was investigated by generating a conditional allele of the Xist gene. A system was set up in which reactivation of two X-linked genes, the endogenous Hprt gene and an X-linked GFP transgene, can be quantitatively assessed. Mouse embryonic fibroblasts derived from mice carrying the conditional Xist mutation were cultured and infected with an adenovirus vector carrying the gene for Cre recombinase. After Cre mediated deletion of Xist, the inactive X remained transcriptionally silent, late replicating, and hypoacetylated on histone H4, confirming that X-inactivation can be maintained in the absence of Xist RNA. However, the Xist mutant inactive X was no longer enriched in histone macroH2A1. Furthermore, the reactivation rate of GFP and Hprt increased, indicating Xist RNA does contribute to gene repression on the inactive X. DNA methylation, histone hypoacetylation and Xist RNA were found to act synergistically in X chromosome silencing.

To investigate whether Xist RNA can also silence the active X chromosome of male somatic cells, Xist expression on the active X was induced by demethylation. Demethylation was achieved by Cre mediated deletion of a conditional mutant allele of DNA methyltransferase-1 (Dnmt1) in male fibroblasts. In these cells, Xist RNA coated the active X chromosome, in a pattern indistinguishable from coating of the inactive X of female cells. Although many Xist expressing chromosomes also transcribed X-linked genes Pgk-1 and Hprt, in a small percent of cells Xist expression led to X chromosome inactivation. The proportion of chromosome expressing X-linked genes declined, and occasionally the X became late replicating, indicating that X-inactivation can be initiated in male somatic cells.

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Chapter 1.

Introduction

DOSAGE COMPENSATION

The evolution of sex chromosomes and chromosomal sex determination in higher organisms necessitated the coevolution of compensation for sex chromosome gene dosage differences. Organisms as diverse as flies, worms, mice and humans compensate for differences in X-linked gene dosage. While the exact mechanism of dosage compensation is different in various organisms, there are some features that are conserved (Cline and Meyer, 1996; Panning and Jaenisch, 1998). First, the onset of dosage compensation is during early embryonic development. Second, dosage compensation is accomplished by large molecular machines that bind the sex chromosome(s) in only one of the sexes. In the fly *Drosophila melanogaster*, a complex containing the MSL proteins and the roX RNAs assemble on the male X chromosome to upregulate gene expression two-fold (Figure 1A) (Meller et al., 2000). In the worm, *Caenorhabditis elegans*, the dosage compensation complex binds the two X chromosomes in female cells and downregulates gene expression by half (Figure 1B) (Chuang et al., 1996). In eutherian mammals, one of the two X chromosomes in females is inactivated in a process that involves a noncoding RNA, Xist in mice and XIST in humans (Panning and Jaenisch, 1998). Xist/XIST most likely is found complexed with proteins, but the identity of the these factors has not been determined (Figure 1C) (Panning and Jaenisch, 1998). A third similarity between dosage
compensation in different organisms is that the process involves remodeling of chromatin to achieve modulation of transcriptional activity. In *Drosophila*, components of the dosage compensation machinery exhibit chromatin remodeling functions, such as ATP-dependent helicase (Lee et al., 1997) and histone acetyltransferase activities (Akhtar and Becker, 2000). The *C. elegans* dosage compensation complex is similar to the Xenopus 13S-condensin complex involved in mitotic chromosome condensation (Meyer, 2000). Dosage compensation complexes probably evolved from existing nuclear complexes that were recruited to the new task of dosage compensation (Pannuti and Lucchesi, 2000).

Although progress has been made in identifying components of the dosage compensation complex, our knowledge of the molecular and biochemical function of the machinery is limited. Functions that the dosage compensation complex must be able to perform include recognition and binding of the X chromosome, but not the autosomes, nucleating and spreading modification of the chromatin over the range of the entire chromosome, and maintenance of dosage compensation from one cell generation to the next. In mammals, another layer of complexity is added to the process, in that the two X chromosomes in the same nucleus are treated differentially by the dosage compensation machinery. This discrimination necessitates some mechanism that distinguishes the two X chromosomes and directs dosage compensation specifically to the inactive X. In this chapter I will review what we know about the mammalian dosage compensation machinery and its mechanism of action, with particular attention paid to the role of Xist RNA in the mouse.

**A historical view**

It was forty years ago that Mary Lyon first proposed the hypothesis that one of two X chromosomes in mammalian females is inactivated at random (Lyon, 1961). The hypothesis was based on observations of variegated phenotype of female mice heterozygous for X-linked coat color mutations. The inactive X was shown to form a
condensed sex chromatin body, so called Barr body, in the nucleus (Barr, 1962). Based on observations that XXX individuals have two Barr bodies and XXXX individuals have three, a modification to the Lyon hypothesis quickly followed that stated that one X chromosome in a diploid nucleus remains active and every other X in excess is inactivated (Lyon, 1962). Hence the idea of the "blocking factor" was born: a factor that specifically inhibits inactivation of one and only one X chromosome per diploid genome (Lyon, 1992). Therefore, in mammals it is the "n-1 rule", and not the ratio of X chromosomes to autosomes, that determines how many X chromosomes will be subject to X-inactivation (Lee and Jaenisch, 1997).

Observations that in X-to-autosomal translocations only one of the two X chromosome fragments is subject to inactivation, gave rise to the idea of an X inactivation center (XIC in humans and Xic in mice) (Russell, 1963). Inactivation was postulated to initiate from this site and spread bidirectionally along the chromosome arms. X chromosome fragments lacking the XIC/Xic are not able to undergo X inactivation (Figure 2). In the mouse, a locus called the Xce (X controlling element) that affects the choice of X chromosome for inactivation, also mapped to the Xic (Johnston and Cattanach, 1981). The discovery of the human XIST and the mouse Xist genes, which map to the XIC/Xic and are uniquely expressed from the inactive X, opened up the field to molecular analysis (Borsani et al., 1991; Brockdorff et al., 1991; Brockdorff et al., 1992; Brown et al., 1991; Brown et al., 1992).

THE MAMMALIAN DOSAGE COMPENSATION MACHINERY

Xist RNA and the X inactivation center

Xist RNA is the only known factor in mammals that specifically binds and regulates the dosage compensated chromosome, analogous to binding of the dosage compensation machinery in flies and worms. Xist lacks any significant protein encoding potential, yet the
RNA is spliced and polyadenylated and is retained in the nucleus (Brockdorff et al., 1992). The gene maps to the X inactivation center (Xic) (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). The Xic regulates several aspects of X-inactivation, including counting the number of X chromosomes in the nucleus, choosing an X chromosome to remain active, and silencing all other X chromosomes (Avner and Heard, 2001). Xist deletion studies demonstrated that the gene is essential for the initiation of silencing, and it plays a role in choice, but it is not necessary for counting (Marahrens et al., 1998; Marahrens et al., 1997; Penny et al., 1996). Accordingly, Xist cDNA transgenes, when induced to express Xist RNA are sufficient for silencing the chromosome, but are not counted as separate Xics (Wutz and Jaenisch, 2000). These observations assign the silencing function of the Xic to the Xist gene, and separate this activity from counting.

However, several lines of evidence indicate that the genomic regions responsible for counting and choice reside in the vicinity of Xist and may act via regulation of Xist expression. A transcript antisense to Xist, Tsix, originates from a CpG island 15 Kb downstream of Xist (Lee et al., 1999a). In undifferentiated cells, both Xist and Tsix are expressed to a low level from all X chromosomes. During differentiation, Tsix expression is turned off and the Xist transcript is stabilized on the X chosen to be inactive. Eventually expression of both Tsix and Xist is turned off on the X chosen to remain active, and differentiated cells transcribe only Xist and exclusively from the inactive X (Figure 3.) (Lee et al., 1999a; Panning et al., 1997; Sheardown et al., 1997). Deletion of the CpG island and the transcription start site of Tsix abolishes choice in that the mutant chromosome is always chosen for X inactivation (Lee and Lu, 1999). That is, a chromosome that does not express Tsix will always express Xist, indicating that Tsix might be an antisense regulator of Xist expression. However, this deletion leaves the counting activity intact and X inactivation is triggered only in cells that contain multiple X chromosomes (Lee and Lu, 1999). Therefore, counting and choice are also genetically separable. On the other hand, a larger 65 kb deletion 3' of Xist abolishes both counting and choice, and the mutant X
chromosome is always inactivated, even in cells with a single X (Clerc and Avner, 1998). These results suggest that the counting element lies within the 65 kb deletion, but outside of the Tsix Cpg island and transcription start site.

Placement of the counting element in the vicinity of Xist is also supported by experiments in which large Xist containing YAC and cosmid transgenes were placed on autosomes of male cells (Herzing et al., 1997; Lee and Jaenisch, 1997; Lee et al., 1996). These transgenes are recognized by the cells as ectopic Xics that trigger counting and lead to expression of the transgenic Xist in some cells and of the endogenous Xist in others. The region of importance was narrowed to 80 kb in one study (Lee et al., 1999b) and 35 kb in another (Herzing et al., 1997); however, the exact position and mechanisms of action of the counting element are unknown. Furthermore, YAC transgenes only appear to function as ectopic Xics when present in multiple copies, indicating that despite their large size, YACs still lack sequences essential for X inactivation (Heard et al., 1999). It is possible that YACs contain all elements necessary for Xic function, but some of these elements, perhaps repetitive sequences, are required in more copies than a single YAC provides. Put together, these results indicate that the Xic may consist of the Xist gene and other elements that regulate its function.

Untranslated RNAs in gene regulation

Xist is not the only noncoding RNA transcribed nearby monoallelically expressed genes. Several imprinted domains, regions of the genome where expression of genes depends on parental origin, also express noncoding RNAs. For example, the H19 RNA shows expression patterns opposite of the nearby imprinted genes Igf2 and Ins2 (Tilghman, 1999). However, no role has been assigned to H19 in regulation of expression of these genes (Jones et al., 1998; Schmidt et al., 1999). Another transcript, Air, is transcribed in the antisense orientation at the imprinted Igf2r locus. The transcript originates from the imprinting box of Igf2r, and it is possible that it is directly involved in the
imprinting process (Wutz et al., 1997). IPW is an gene encoding another untranslated RNA that is located in the region deleted in Prader-Willi syndrome (Wevrick and Francke, 1997; Wevrick et al., 1994). The RNA may be involved in regulating imprinted gene expression in the region, and its lack may contribute to the phenotypes of the Prader-Willi syndrome. Another noncoding RNA termed NTT has been isolated in T-cells (Liu et al., 1997). All these noncoding RNAs are retained in the nucleus and localize to their site of transcription. Whether they are functionally involved in gene regulation, or are simply the byproducts of open chromatin is unclear. Xist is the best studied example of noncoding RNAs and it may represent a novel mechanism used by many unconventional RNAs to repackage local chromatin and regulate expression of genes.

**Xist, a functional RNA, as part of a putative dosage compensation RNP complex**

Xist RNA is over 15 kb long and it lacks an open reading frame of significant length, suggesting that the gene may instead encode a functional RNA (Brockdorff et al., 1992). A detailed study of the localization of Xist RNA in female nuclei provides further evidence for this idea (Clemson et al., 1996 and see Figure 4.). Xist RNA occupies the same three dimensional nuclear territory as the inactive X chromosome, painting its entire surface. It was proposed that Xist RNA acts either via altering the chromosome architecture of the inactive X or via setting up a specialized nuclear compartment. The latter idea is supported by the observation that the association of Xist RNA and the chromosome appears not very tight. Xist is found in the nuclear matrix fractions and remains in the nucleus even after extraction of chromosomal DNA and histones (Clemson et al., 1996). Further evidence for the functional nature of the RNA comes from Xist deletion and transgene studies. An Xist deletion that leaves the promoter intact and therefore allows transcription of the truncated RNA is nevertheless a functional null allele, indicating that transcription through the region is not sufficient for Xist function (Marahrens et al., 1997).
On the other hand, a small Xist transgene, containing just the cDNA separated from all endogenous regulatory sequences can reversibly silence the chromosome of undifferentiated cells when induced to express Xist RNA (Wutz and Jaenisch, 2000).

Since Xist does not seem to encode a protein and instead acts as a functional RNA, several attempts were made to identify sequences within the gene that can potentially define functional domains. A region containing a tandem repeat sequence at the 5' end of the gene (Xcr) was identified as one of the most conserved features of Xist genes from several different mammalian species (Hendrich et al., 1993). This repeat region is able to silence X-linked genes in an in vitro transient transfection assay (Allaman-Pillet et al., 2000). More importantly, the silencing function of the Xcr repeats was shown in a chromosomal context. An Xist transcript expressed from a cDNA transgene that lacks the Xcr sequences can localize to the chromosome but cannot silence it (Wutz, A. and Jaenisch, R. in preparation), indicating that the "silencing domain" of the RNA maps to this region. Xist RNA in differentiated cells is transcribed from two different promoters. The P1 promoter gives rise to about 10-20% of the total Xist transcripts, and a downstream P2 promoter gives rise to the major portion, 70-90% (Johnston et al., 1998; Memili et al., 2001). Interestingly, P2 Xist transcripts lack the Xcr sequences and hence the putative silencing domain (Memili et al., 2001). It is possible that two pools of Xist RNA exist in female somatic cells: P1 transcripts that are directly involved in X chromosome silencing and P2 transcripts that are involved in X inactivation in some other way, for example by playing a structural role.

Although Xist interacting proteins have not been found yet, with the possible exception of heteronuclear protein hnRNPC1/C2 (Brown and Baldry, 1996), it is widely believed that Xist associates with other factors and forms a ribonucleoprotein complex. Ribonucleoprotein complexes acting as molecular machines are not unprecedented as the ribosome, the spliceosome and telomerase all have both protein and RNA components. The RNA component in these complexes plays more than a simple structural role. In fact, in
the case of the ribosome, the protein component appears to be the structural unit and the RNA component the enzymatically active one. The active site of the ribosome is devoid of protein and the key peptidyl transferase reaction is accomplished by the RNA component (Ban et al., 2000; Nissen et al., 2000). Similarly, Xist RNA is likely to be a key component of the dosage compensation machinery.

The low level of Xist RNA detected in undifferentiated cells appears to consist of unstable transcripts, while Xist RNA is highly stable in somatic cells (Panning et al., 1997; Sheardown et al., 1997). These observations led to the suggestion that at the onset of differentiation and X-inactivation, Xist RNA associates with stabilizing factors, resulting in an RNP complex (Panning et al., 1997; Sheardown et al., 1997). This putative complex is analogous to the dosage compensation complex of Drosophila that consist of the MSL proteins and the roX1 and roX2 RNAs. The roX RNAs are highly unstable in the absence of the MSL proteins, implying that binding to the MSL proteins may be necessary to protect the RNAs from degradation (Meller et al., 2000). In the case of Xist, the factors that stabilize the RNA are not the same as the ones that localize the complex to the inactive X, as these two events can be separated (Clemson et al., 1998). The particulate appearance of the Xist RNA signal in fluorescent in situ hybridization (FISH) experiments is consistent with the RNA being complexed with other factors (Clemson et al., 1996; Panning and Jaenisch, 1996; Panning and Jaenisch, 1998) (Figure 4.).

The recent discovery that chromodomains can interact with RNA (Akhtar et al., 2000) provides some clues as to where to look for Xist interacting proteins. The chromodomain is a motif commonly found in several heterochromatin associated proteins that act as transcriptional repressors. Some proteins that have a transcriptional activator function also contains chromodomains, such as components of the Drosophila dosage compensation complex: MSL-3 and MOF. The chromodomains of MSL-3 and MOF were found to interact with RNA; specifically the MOF chromodomain binds roX2, the noncoding RNA involved in Drosophila dosage compensation (Akhtar et al., 2000). By
analogy, mouse chromodomain containing proteins may interact with Xist RNA as part of the mammalian dosage compensation complex (Figure 1.). However, chromodomains have also been shown to bind with high affinity to methylated lysine residues of histone H3, indicating that different chromodomains may have different functions (Bannister et al., 2001; Lachner et al., 2001).

Recognition of the X, and nucleation and spreading of dosage compensation

The dosage compensation complex needs to be able to recognize the X and distinguish it from autosomes. The complex then nucleates formation of the dosage compensated chromatin, which then spreads along the length of the chromosome and is subsequently maintained. The molecular mechanism of these processes is not well known, especially in mammals, but important lessons can be learned from comparative analysis of dosage compensation in Drosophila and C. elegans. In mammals, as in Drosophila, noncoding X-linked RNAs provide the unique capacity to mark the X, as the RNA can be localized to its site of transcription. Both in mammals and in Drosophila, Xist and the roX RNAs respectively, have been shown to coat the chromosome from which they are transcribed (Clemson et al., 1996; Meller et al., 1997). Nucleation of the dosage compensation complex at the appropriate site can be easily accomplished by limiting assembly of the complex to the sites of RNA synthesis. Similar noncoding RNAs have not been found in C. elegans. In this organism, the SDC-2 protein seems to be responsible for recognizing the X and recruiting the dosage compensation complex to the chromosome (Dawes et al., 1999). However, the sites of SDC-2 binding and of dosage compensation nucleation are not known.

Current models favor the view that once the complex is assembled, dosage compensation is initiated by local alterations of the chromatin which then spreads to neighboring regions in cis. Whether additional sequences are required for efficient
spreading of dosage compensation can be determined by analyzing X-autosomal translocations and transgenes containing dosage compensation nucleation sites integrated into autosomal material. In *C. elegans*, a single example of X-autosomal translocation has been described in which the dosage compensation complex bound to the X portion of the hybrid chromosome but not the autosomal material, indicating that X chromosome sequences are required for spreading of dosage compensation (Lieb et al., 2000). By contrast, in Drosophila, roX RNAs can spread to other distant chromatin entry sites that provide additional sites for assembly of the dosage compensation complex in a stepwise process (Meller et al., 2000). The RNAs can even travel *in trans*, from a roX1 transgene located on an autosome to the nontransgenic homologue and to the X (Kelley et al., 1999). Of the about 35 chromatin entry sites scattered along the Drosophila X, two are sites of roX1 and roX2 synthesis, and the identity of the other sites is not known. From these chromatin entry sites, dosage compensation can spread hundreds of kilobases into flanking chromatin (Kelley et al., 1999). It has been postulated that dosage compensation is regulated on the level of chromosom al domains, with each domain being under the influence of a chromatin entry site (Kelley and Kuroda, 2000; Kelley et al., 1999).

The difference between spreading of dosage compensation in Drosophila and in mammals is that in the latter spreading only initiates from the site of Xist synthesis (there is only one chromatin entry site) and spreading is strictly limited *in cis* (Avner and Heard, 2001). Restricting dosage compensation *in cis* is essential, as the two X chromosomes in the female nucleus must be discriminated, and spreading of inactivation *in trans* would be lethal. In mammals, dosage compensation is able to spread along the length of the entire chromosome from a single nucleation sites. If the site of Xist synthesis is moved to an autosome, dosage compensation can spread also along the entire length of the autosome (Lee and Jaenisch, 1997; Wutz and Jaenisch, 2000). However, spreading is attenuated into some autosomes, indicating that although not essential for spreading, the X chromatin somehow is more favored by the machinery (Lyon, 1998b).
To explain variable spreading of X-inactivation into autosomes, Riggs proposed that "booster stations" along the X chromosome facilitate spreading of inactivation (Riggs, 1990). Later Mary Lyon argued that repetitive LINE-1 (L1) elements may act as booster stations (Lyon, 1998a). This idea is based on observations that the human and mouse X chromosomes stain brightly with LINE-1 probes in fluorescent in situ hybridization experiments, indicating that LINE-1 elements on this chromosome are more densely distributed (Boyle et al., 1990). Furthermore, autosomes refractory to the spread of dosage compensation are less LINE-1 rich (Lyon, 1998a; Lyon, 1998b). These results were later confirmed by analyzing sequence data from the Human Genome Project. This analysis also showed that L1 density on the X is greatest around the X inactivation center, and regions of the human X chromosome that escape X inactivation are relatively L1 poor (Bailey et al., 2000). The Drosophila X chromosome is similarly enriched in short and long repetitive sequences (DiBartolomeis et al., 1992; Lowenhaupt et al., 1989; Pardue et al., 1987; Waring and Pollack, 1987), and it has been proposed that these repeats may help spreading of dosage compensation from chromatin entry sites also in this organism (Kelley et al., 1999).

LINE-1 elements may facilitate the spread of heterochromatinization on the X by providing binding sites for Xist RNA and the dosage compensation complex (Lyon, 1998a). It is relevant in this context that estimates of the number of Xist RNA molecules in female nuclei indicate that there is not enough RNA to coat the entire chromosome by binding to it processively (Buzin et al., 1994), rather Xist might bind numerous yet distinct sites along the chromosome. Analysis of localization of Xist RNA on rodent metaphase chromosomes also indicates that binding is nonuniform, with preferential association with G-light bands (Duthie et al., 1999). Consistent with this LINE-1 elements are enriched in G-light bands on both mouse and human X chromosomes (Bailey et al., 2000; Boyle et al., 1990). However, dark G bands are equally L1 rich (Bailey et al., 2000; Boyle et al., 1990), indicating that if L1s are indeed Xist binding sites, not all L1s are treated equally.
An alternative model for the role of LINE-1 elements in X chromosome inactivation was proposed by Marahrens (Marahrens, 1999). He reasons that X-inactivation may be initiated by physical pairing of the homologous X chromosomes, in a process analogous to transvection in nonmammalian organisms. Transvection is defined as changes in gene expression as a result of physical interaction between homologous regions (Henikoff and Comai, 1998). The spread of heterochromatinization then may be achieved by repeat induced silencing facilitated by interspersed LINE-1 elements (Lyon, 1998a; Marahrens, 1999; Wolfe, 1997).

Any model for spreading of dosage compensation must take into account that X chromosome inactivation is not contiguous. Many genes have been identified that escape X-inactivation, especially on the human X chromosome (Carrel et al., 1999; Disteche, 1995). Mouse homologues of most human genes that escape X inactivation are subject to silencing, indicating evolutionary differences between the X chromosomes (Tsuchiya and Willard, 2000). The clustering of many escapees on the human chromosome (Miller and Willard, 1998) raises the possibility that the X chromosome is regulated on the level of chromosome domains, perhaps defined by matrix attachment regions (Disteche, 1999). However, a more refined, perhaps gene specific control must be superimposed on domain regulation, as some genes that escape inactivation are found to be interspersed between repressed genes (White et al., 1998). It is also possible that two classes of genes escape silencing: one class that resists the original X inactivation signal and another that is first silenced than is reactivated. These two classes may be subject to regulation on different levels.

FEATURES OF THE INACTIVE X CHROMATIN

Once X inactivation has been established, the mammalian inactive X acquires several features, such as condensation of chromatin, late replication timing, hypoacetylation
on histone H4, enrichment in histone macroH2A1, and methylation on CpG islands (Heard et al., 1997). It is clear that initiation of X-inactivation by an Xist RNA mediated mechanism is necessary to trigger formation of the inactive X chromatin; however, very little is known about the targeting of these chromatin remodeling activities to the inactive X.

**Higher order chromosome structure**

X chromosome inactivation ultimately results in a altered higher order structure of the chromosome. The condensational and conformational differences of the inactive X are more likely a consequence of the inactivation process rather than a mechanism causing inactivation to take place. The cytologically visible differences between the active and inactive X chromosomes include condensed appearance in interphase and formation of the Barr body (Barr, 1962), and the presence of a bend in mitotic inactive X chromosomes (Flejter et al., 1984). While the condensed appearance of the Barr body and would suggest a higher nucleosome concentration and more condensed chromatin, optical sectioning of stained chromosomes failed to detect volume differences between the active and inactive X chromosomes (Eils et al., 1996).

**Replication timing**

Delayed replication timing is perhaps the most universal characteristic of silenced genes. The inactive X chromosome as a whole and individual genes on the chromosome replicate late in S phase (Hansen et al., 1996; Priest et al., 1967; Schmidt and Migeon, 1990; Torchia et al., 1994). However, it is not clear whether delayed replication timing is the cause or consequence of condensation of inactive chromatin. The timing of DNA replication is regulated at the level of initiation at origins of replication. There is experimental evidence in support of the idea that delayed replication timing can be caused by localization of origins of replication in heterochromatic regions of the genome (Pak et al., 1997; Wintersberger, 2000). Similarly, aberrant replication timing can lead to defective
chromosome condensation, indicating that proper regulation of replication timing is important for the correct assembly of chromatin (Loupart et al., 2000). It is possible that the pool of chromosomal proteins is different in the early and late stages of S phase and chromatin assembled at different times would thus have a different structure. If delayed replication interferes with transcription and transcriptional inactivity delays replication, a positive feedback mechanism is set up that maintains the silent state.

**Hypoacetylation of core histones**

Modification of the amino terminal tails of core histones provides a way to alter the basic structure of the nucleosome and hence remodel the chromatin. The level of histone acetylation on amino terminal lysines is determined by the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kouzarides, 2000). Histone acetylation plays an important role in gene regulation, with acetylation correlating with gene activity and deacetylation with repression (Grunstein, 1997). Several activators and repressors have been shown to act via recruiting HATs or HDACs respectively (Brownell and Allis, 1996; Pazin and Kadonaga, 1997). The entire inactive X chromosome is underacetylated on histones H2A, H3 and H4 (Belyaev et al., 1996; Jeppesen and Turner, 1993). Underacetylation of histone residues is a conserved feature of the inactive X between eutherian and marsupial mammals (Wakefield et al., 1997). Furthermore, alteration in histone acetylation levels is also involved in Drosophila dosage compensation, where MOF (a histone acetyltransferase) is specifically responsible for acetylation of histones on the dosage compensated chromosome of males (Akhtar and Becker, 2000).

The functional importance of histone deacetylation in gene silencing has clearly been demonstrated using inhibitors of histone deacetylases Trichostatin A (TSA) and Sodium butyrate (Yoshida et al., 1995). The inactive X chromosome becomes globally underacetylated during differentiation of embryonic cells (Keohane et al., 1996). If embryonic stem cells are differentiated in the presence of TSA, the X chromosome remains
acetylated, despite becoming late replicating and therefore inactive (O'Neill et al., 1999). However, once the inactive X is hypoacetylated in somatic cells, histone deacetylase inhibitor treatment does not increase levels of histone acetylation on the chromosome (Jeppesen and Turner, 1993). Accordingly, it was shown that the rate of acetate turnover greatly decreases as cells undergo differentiation (Keohane et al., 1998). Therefore, a histone deacetylase activity is necessary for establishment of an underacetylated chromosome, but is less important for maintenance. The identity of the enzyme(s) responsible for deacetylation of the inactive X chromosome is not known.

Originally it was believed that acetylation of histones opens up chromatin by neutralizing the positive charge of lysine residues and hence making interactions with the negatively charged DNA less favorable (Brownell and Allis, 1996). Deacetylation of histones would close the chromatin again making it inaccessible to transcription factors (Pazin and Kadonaga, 1997). The recent discovery that bromodomains specifically interact with acetylated lysines opens up another possibility that acetylation serves to recruit a new family of proteins and alter chromatin structure via binding of these proteins (Dhalluin et al., 1999; Jacobson et al., 2000). Bromodomains are commonly found in several chromatin associated proteins, such as eukaryotic transcription factors and nuclear histone acetyltransferases, either singly or in pairs (Winston and Allis, 1999). An acetylated histone-bromodomain interaction could anchor HATs to acetylated chromatin and recruit other bromodomain containing proteins to further stabilize the complex (Figure 1.) (Winston and Allis, 1999).

**DNA methylation**

DNA methylation has long been considered an important mechanism stabilizing the inactive state of the eutherian inactive X chromosome (Heard et al., 1997). DNA methylation in general correlates with transcriptional silencing and is also involved in regulation of imprinted genes and perhaps tissue specific genes as well (Jaenisch, 1997).
Global DNA methylation profiles are set up by the de novo methyltransferases Dnmt3a and Dnmt3b (Okano et al., 1999; Okano et al., 1998), and maintained by Dnmt1 (Li et al., 1992). The preferred site of methylation is CpG dinucleotides. While all three enzymes are essential during development, the most severe phenotype is exhibited by Dnmt1 mutants (Li et al., 1992; Okano et al., 1999). In these mutants, methylation set up by Dnmt3a and Dnmt3b cannot be maintained and it is progressively lost during successive rounds of cell division.

As methyl-cytosine can easily mutate to thymine (Bird, 1980; Coulondre et al., 1978), mammalian genomes are depleted of CpG dinucleotides, except in regions that are specifically demethylated, such as promoter regions of genes. These relatively CpG rich regions are called CpG islands (Bird, 1986). CpG islands are almost never methylated, one exception being CpG islands on the inactive X. Corresponding CpG islands on the active X are unmethylated (Norris et al., 1991). While the global methylation level of the X chromosomes is controversial (Norris et al., 1991), differential CpG island methylation in the active and inactive X chromosomes is well established. Human females suffering from ICF syndrome (immunodeficiency, centromeric instability, facial anomalies) have a mutation in DNMT3B and lack CpG methylation on the inactive X chromosome (Hansen et al., 2000). This observation indicates that Dnmt3b/DNMT3B may be specifically responsible for methylating inactive X-linked CpG islands.

The importance of DNA methylation in silencing X inactivated genes has been demonstrated in experiments in which repressed genes were reactivated using 5-azadC (5-azadeoxycytidine) or 5-azaC (5-azacytidine) (Graves, 1982; Mohandas et al., 1981). These drugs are nonmethylatable cytosine analogues, and their incorporation into DNA leads to demethylation (Santi et al., 1983). Moderate doses of these drugs reactivate genes on the inactive X at low frequency, providing evidence for the stabilizing role methylation plays in X chromosome inactivation. However, it is possible to silence the X chromosome in the absence of CpG methylation, as it is done in marsupials (Kaslow and Migeon, 1987;
Loebel and Johnston, 1996), in *Dnmt1* mutant differentiating embryonic stem cells (Panning and Jaenisch, 1996), or on the inactive X of ICF patients (Hansen et al., 2000). Furthermore, X inactivation in extraembryonic tissues of eutherian mammals can tolerate extensive demethylation, indicating that methylation is not an essential feature of X-linked gene repression in these tissues (Sado et al., 2000). Indeed, DNA transfection studies suggest that methylation may actually be absent from the inactive allele of *Hprt* in the extraembryonic tissues of mice (Kratzer et al., 1983). This conclusion is based on the observation that inactive X DNA isolated from the yolk sac endoderm is functional in gene transfer experiments, while the inactive X DNA from the embryo proper is not. However, silencing in the absence of methylation in marsupials, the extraembryonic tissues of mice, and in cells of ICF patients is relatively unstable.

Methylation of DNA is a mechanism of dosage compensation and gene repression in mammals that is not shared with lower organisms. It is likely added as another layer of stability of inactivation, necessitated by the longer lifespan of the organism. Consistent with that, methylation of the *Hprt* gene takes place several days after X chromosome inactivation, and therefore it is generally believed that methylation plays a stabilizing role rather than being involved in the initiation of X-inactivation (Lock et al., 1987).

In recent years, significant progress has been made in elucidating the mechanism by which methylation inhibits transcription. The original idea was that methylation sterically hindered binding of transcription factors to promoter regions. However, recently a family of methyl-DNA binding proteins has been identified. Several of these proteins act as transcriptional repressors by interacting with histone deacetylases, recruiting them to their binding sites and altering the structure of chromatin and its accessibility (Figure 1.). For example, the transcriptional repressor domain of methyl-DNA binding protein MeCP2 has been shown to interact with a corepressor complex that contains mSin3A and histone deacetylases (Jones et al., 1998; Nan et al., 1998). Furthermore, methyl-DNA binding protein MBD2 is part of another complex, MeCP1, that also contains histone deacetylase
HDAC1 (Ng et al., 1999). Repression by MBD1 is also sensitive to histone deacetylase inhibitors, indicating that its mechanism of repression is likely to involve a histone deacetylase (Ng et al., 2000). MBD3 also contains methyl-DNA binding domain, but it does not directly bind DNA. However, it was found to be part of the NuRD complex that contains nucleosome remodeling and histone deacetylase activities (Zhang et al., 1999). Moreover, Dnmt1 itself has been shown to associate with histone deacetylases (Fuks et al., 2000) and therefore binding of methyl-DNA binding proteins may not always be necessary for recruitment of histone deacetylases to methylated DNA. Thus DNA methylation leads to assembly of specialized chromatin containing methyl-DNA binding proteins and deacetylated histones.

Histone macroH2A1

Histone macroH2A1 and macroH2A2 are unusual core histone variants with a large nonhistone domain of unknown function (Pehrson and Fried, 1992). When female cells are stained with antibodies against these histones, an intensely staining domain, the macrochromatin body (MCB), is visualized that colocalizes with the inactive X (Costanzi and Pehrson, 1998; Costanzi and Pehrson, 2001). Therefore, it was proposed that the inactive X chromatin is enriched in histone macroH2A1 and macroH2A2. Recently, this conclusion has been questioned, however, by studies in which it was shown that a similarly brightly staining chromatin body, colocalizing with the Barr body, can be seen after staining female cells with antibodies against other core histones (Perche et al., 2000). The authors argued that the intense staining is a consequence of higher nucleosome concentration not of altered histone content. A shortcoming of this study is that the experiments were performed in cell types with an easily visible Barr body and the histone antibody staining correlated with the DAPI counterstain. However, intense macroH2A1 staining on the inactive X can be seen in cells lacking a well defined Barr body, and in
metaphase chromosome spreads as well, and in these cases macrohistone staining is not simply mirroring the DNA counterstain (Costanzi and Pehrson, 1998). Nevertheless, this issue needs to be resolved. Recently, another histone variant distantly related to histone macroH2A, H2A-Bbd was identified (Chadwick and Willard, 2001). The inactive X chromatin appears deficient in this histone, while the autosomes and the active X are not (Chadwick and Willard, 2001). This observation raises the possibility that macroH2A1/2 is preferentially found in inactive chromatin, and H2A-Bbd in active chromatin (Figure 1).

Intense macroH2A1 staining has also been observed in other tissues where the X chromosome is inactivated, such as in the XY compartment of male meiotic nuclei (Hoyer-Fender et al., 2000; Richler et al., 2000) and the trophectodermal cells of female preimplantation blastocysts (Costanzi et al., 2000). By contrast, in undifferentiated ES cells, prior to the onset of X-inactivation, macroH2A1 localizes to the centrosomes (Rasmussen et al., 2000) away from chromatin (Mermoud et al., 1999). These results suggest that the centrosome might be involved in loading of macroH2A1 to the X chromosome at the onset of X-inactivation.

The functional involvement of histone macroH2A in repression of X-linked genes, or the mechanism of this repression, has not been established. The large nonhistone domain of the protein may recruit transcriptional repressors to inactive chromatin. Transient transfection experiments suggests that this domain can repress transcription if tethered to the promoter region of reporter genes via a Gal4 DNA binding domain (Perche et al., 2000). However, the function of a core nucleosomal histone can be different on naked DNA than on assembled chromatin. To fully assess the in vivo function of macroH2A1, mutational inactivation of the gene will be necessary.

**REACTIVATION OF X-LINKED GENES**

Attempts to reactivate the inactive X chromosome are almost as old as Lyon's X-inactivation hypothesis. True reversal of X-inactivation occurs during normal development
in oogenesis in female mammals (Handel and Hunt, 1992). There is also evidence showing that inactivation of the X chromosome becomes less stable as the organism ages (Wareham et al., 1987). To understand the molecular mechanisms behind X-inactivation, numerous attempts have been made to induce reactivation experimentally (Gartler and Goldman, 1994). The most successful chromosomewide reactivation experiments involve fusion of female somatic cells with less differentiated ones (Takagi, 1983; Takagi, 1988; Takagi, 1993; Takagi et al., 1983). Reactivation is likely related to the differentiation status of the hybrid cell (Takagi, 1988). Perhaps undifferentiated cells contain cytoplasmic factors that can reverse formation of the inactive X chromatin. Similar conclusions can be drawn from recent nuclear transfer experiments in which female somatic cell nuclei are moved into the oocyte cytoplasm. During the cleavage stages of development of these cloned embryos, both X chromosomes are active, indicating that factors in the oocyte cytoplasm are able to reprogram epigenetic marks on the active and inactive X chromosomes (Eggen et al., 2000).

The reactivation frequency of X-inactivated genes is higher than normal also in hybrid cells resulting from cell fusion between two different differentiated cell types, not only in cell resulting from fusion involving undifferentiated cells. In these hybrids, increase in reactivation cannot be attributed to the undifferentiated status of one of the partners. Rodent-human hybrids generally retain a full complement of rodent chromosomes and but lose many human chromosomes. Hybrids that retain the human inactive X as the sole human component are useful in studying the process of X-inactivation. However, silencing of the human inactive X in these cells appears less stable, which may be related to species specific differences in the factors required for efficient maintenance of silencing. Indeed, the interphase structure of the human inactive X appears less condensed and the chromosome is less peripheral in the nucleus of these hybrids (Dyer et al., 1989). The human XIST RNA also appears to be localized abnormally in these hybrids: XIST RNA
signals are more diffuse and do not perfectly colocalize with the X chromosome (Clemson et al., 1998; Hansen et al., 1998).

Many attempts at reactivating the inactive X were made by inhibiting silencing mechanisms in somatic cells. Patchy reactivation of one or a few genes have been seen at a low frequency. Most studies used nonmethylatable cytidine analogs 5-azadC or 5-azaC to inhibit methylation and they are reviewed in (Gartler and Goldman, 1994). Coordinate reactivation of three genes within 100 kb of each other has been seen, raising the possibility that the X chromosome is regulated in units of chromosomal domains (Toniolo et al., 1988). However, this conclusion remains speculative. Reactivating a gene on a chromosome using 5-azadC also increases the probability that other distant genes will be reactivated, although the probability remains modest (Ellis et al., 1987; Hors-Cayla et al., 1983; Mohandas et al., 1981; Schmidt et al., 1985). This observation indicates a certain degree of cooperativity in silencing of genes on the X chromosome by methylation.

THE ROLE OF XIST IN THE MAINTENANCE OF THE INACTIVE CHROMATIN CONFIGURATION

Several studies have shown that once X-inactivation has been established, the Xic is not required to maintain the chromosome in an inactive state. One study used mouse-human somatic cell hybrids that were selected for breakage of the human inactive X in such a way the XIC is removed (Brown and Willard, 1994). The other study analyzed leukemia cells containing rearranged isodicentric inactive X chromosomes formed by the fusion of two identical inactive X fragments, both containing a centromere (Rack et al., 1994). Although these studies clearly showed that X-inactivation can be maintained in the absence of the Xic, a quantitative assessment of the role of Xist in somatic cells remained to be done. To be able to delete Xist from the inactive X chromosome of somatic cells, we generated a conditional allele of the gene using the Cre-loxP system (Chapter 2). Following Cre mediated deletion of Xist mouse embryonic fibroblasts, the inactive X
chromosomes remained transcriptionally silent, late replicating, and underacetylated on histone H4, confirming earlier studies. The easiest interpretation of these results is that after X-inactivation has been established by an Xist RNA mediated mechanism, independent mechanisms are recruited to stabilize the inactive state. In a study by Wutz and Jaenisch (2000), the timing of these events was determined using embryonic stem cells expressing an inducible Xist cDNA transgene. During the initial 48 hours of differentiation, Xist RNA initiates X inactivation, but silencing is reversible. After 48 hours, X-inactivation becomes irreversible and Xist independent, presumably because methylation, late replicating and histone hypoacetylation locks in the silent state. This conclusion is consistent with observations that both the appearance of hypoacetylated chromosomes and methylation of CpG islands appear to be late events in X inactivation and therefore are more likely to be involved in maintenance than in establishment (Keohane et al., 1996; Lock et al., 1987). Stabilizing the inactive state by multiple mechanisms may be necessary given the multicellular nature and long lifespan of mammalian species and the devastating consequences of even small anomalies in dosage compensation (Migeon et al., 1994).

Despite the fact that the inactive state can be efficiently maintained in the absence of Xist, the RNA continues to coat the chromosome for duration of the organism's life, suggesting that it continues to play a role in X inactivation (Clemson et al., 1996). In Chapter 3, a system is set up in which rare reactivation events can be assessed quantitatively. These experiments provide direct evidence for the continued involvement of Xist in X chromosome silencing. Therefore, the mechanism mediated by Xist not only triggers recruitment of independent silencing mechanisms to the X at the onset of X inactivation, but is itself responsible for silencing. Silencing by Xist appears similar to gene repression by DNA methylation in that genes are reactivated individually, yet reactivation of one increases the probability that another on the same chromosome will also be reactivated. Xist RNA is capable of silencing the chromosomes independently of other mechanisms, as evidenced by silencing of genes in undifferentiated cells without the chromosome becoming
late replicating or hypoacetylated (Wutz and Jaenisch, 2000). However, when these other silencing mechanisms are present, such as in somatic cells, Xist RNA, DNA methylation, and histone hypoacetylation act synergistically to achieve the remarkable stability of X-inactivation.

In chapter 4, the process of initiation of X-inactivation is revisited: Xist RNA expression is induced by demethylation in fully differentiated cells and we show that Xist RNA is able to initiate X-inactivation outside of the normal time window of initiation that occurs during early differentiation. Therefore, Xist RNA is not only necessary for wild type levels of gene repression on the inactive X of female somatic cells, it is also sufficient to induce de novo X-inactivation. However, silencing is seen in only a few percent of cells, consistent with observations of other groups that Xist is unable to initiate X-inactivation as efficiently in somatic cells as in cell undergoing differentiation (Clemson et al., 1998; Tinker and Brown, 1998).

What might then be the function of Xist and its associated proteins? The Drosophila dosage complex contains the enzyme MOF responsible for bringing about the male X specific histone acetylation (Akhtar and Becker, 2000). In mammals, no such activity has been attributed to the Xist containing RNP complex. In Chapter 2, Xist RNA is shown to be essential for the localization of macroH2A1 to the inactive X, providing the first clue as to the mechanism of Xist RNA mediated silencing. Xist RNA is also sufficient for the localization of histone macroH2A1 and thus for the formation of an MCB on the chromosome, as long as the cells are undergoing or have undergone differentiation (Rasmussen, T., Wutz, A., Pehrson, J.R., and Jaenisch R., submitted). It has been suggested that Xist RNA interacts with histone macroH2A1 directly, based on a region of homology to an RNA binding motif in the nonhistone domain of macroH2A1 (Pehrson and Fuji, 1998). Whether the interaction with macroH2A is direct or indirect, these results strongly implicate Xist in the formation of the specialized inactive X chromatin. Recently, it was suggested that the observed bright staining of the MCB with histone macroH2A1
antibodies is a consequence of chromosome condensation, not of altered histone content (Perche et al., 2000). If that is the case, Xist RNA would have to be involved in determining the level of condensation of the chromosome.
REFERENCES


**Figure 1.** The dosage compensation machinery of flies (Cline and Meyer, 1996; Meller et al., 2000), worms (Meyer, 2000) and mice and modifications of the dosage compensated chromatin. The X chromosome of the dosage compensated sex is indicated by a box.

(A) In *Drosophila*, the roX RNAs nucleate assembly of the dosage compensation complex on the male X chromosome. The chromodomains of MSL-3 and MÔF bind to the RNA, followed by recruitment of other MSL proteins. MOF acetylates lysine-16 of histone H4 (H4-Ac), facilitating hypertranscription of genes.

(B) In *C. elegans*, the SDC-2 protein recruits other members of the dosage compensation complex to the X chromosomes of hermaphrodites. The complex that assembles on the chromosomes is similar to the *Xenopus* 13S-condensin complex involved in mitotic chromosome condensation, suggesting that dosage compensation in the worm may involve inducing chromosome condensation in interphase to downregulate gene expression by half.

(C) In mice, dosage compensation is initiated by Xist RNA on one of the X chromosomes in female nuclei. Xist RNA presumably binds other factors, perhaps chromodomain containing proteins (chromo) to inactivate the chromosome. Once X-inactivation is established, the inactive chromatin is hypoacetylated on histone H4 (H4), and methylated on CpG islands (CH3). The inactive X is enriched in histone macroH2A1 and macroH2A2 (macroH2A1/2), while the active X is enriched in histone H2Bbd (H2Bbd). These chromatin modifications may serve to recruit other factors. Methyl-DNA binding proteins (MBD) can bind methylated CpG sites and recruit histone deacetylases (HDAC) to the inactive X. On the active X, acetylated histones may recruit bromodomain containing proteins (bromo).
Figure 2. X-autosomal translocations and the location of the X inactivation center. The X chromosome fragment containing the Xic is subject to inactivation, while the fragment separated from the Xic is not. Chromosomal inactivation spreads bidirectionally from the Xic (red arrow), and can spread into autosomal material attached to the Xic containing X chromosome fragment to a variable degree.
Figure 3. Expression of Xist and Tsix during cellular differentiation. In undifferentiated cells low levels of both Xist (red) and Tsix (green) are expressed from both Xs of female cells and the single X chromosome of male cells. As cells begin to differentiate, Xist RNA is stabilized and Tsix expression is turned off on the future inactive X of female cells. Xist RNA coats the entire inactive X. The future active X continues to transcribe low levels of both Xist and Tsix for some time. In fully differentiated cells, Xist and Tsix expression is turned off on the active X, and Xist continues to be expressed solely from the inactive X.
Figure 4. Localization of Xist RNA in female nuclei.
(A) RNA fluorescent in situ hybridization assay shows that the Xist RNA signal (red) localizes to a specific site in the nucleus and is highly particulate in nature. (B) Xist RNA (red) coats one of the two X chromosomes (green) in female nuclei. (C) The mouse Xist RNA (red) continues to coat the length of the X chromosome through most of mitosis. Methods are described in Chapter 2.
Chapter 2.

Deletion of Xist disrupts histone macroH2A1 localization but not maintenance of X-inactivation

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Data presented in this chapter have been published in Nature Genetics (1999) 22:323-4

Respective contributions: John R. Pehrson provided the antibodies to histone macroH2A1; Barbara Panning and Brian Bates introduced the adenovirus system, FISH technology and the Cre-loxP system in our lab.

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ABSTRACT

To study the role Xist RNA plays in the maintenance of X-inactivation, we generated a conditional mutation in the gene using the Cre-loxP system. Embryonic fibroblasts were derived from mice carrying the conditional Xist allele. Following Cre mediated excision of Xist, the inactive X of female fibroblasts remained transcriptionally silent, late replicating, and hypoacetylated on histone H4. Our results confirm earlier studies that indicated that X chromosome inactivation can be maintained in the absence of Xist RNA. However, histone macroH2A1 was no longer concentrated on Xist mutant inactive X chromosomes, indicating that increased macroH2A1 content is not required to maintain the silent state. However, loss of Xist RNA and histone macroH2A1 enrichment may lead to decreased stability of silencing.

INTRODUCTION

In mammalian females dosage compensation is achieved by inactivating one of two X chromosomes. The inactive X chromosome resembles constitutive heterochromatin several ways. It remains condensed in interphase (Barr, 1962), replicates late in S phase (Priest et al., 1967), is methylated on CpG islands (Norris et al., 1991), and is hypoacetylated on histone H4 (Jeppesen and Turner, 1993; Keohane et al., 1996). Moreover, the inactive X is enriched in histone macroH2A1 (Costanzi and Pehrson, 1998), an unusual variant of histone H2A that has a large nonhistone domain and a domain that closely resembles a full length H2A (Pehrson and Fried, 1992). The inactive X also transcribes Xist (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991), a nuclear, untranslated RNA (Brockdorff et al., 1992) that coats the chromosome in cis (Clemson et al., 1996).
The *Xist* gene is essential for the initiation of X-inactivation (Marahrens et al., 1997; Penny et al., 1996), but no role has been assigned to it in maintenance. Continued transcription of *Xist* RNA and its close association with the inactive X suggests a role for the RNA in differentiated cells (Clemson et al., 1996). Yet, previous studies showed that a fragment of the human inactive X lacking the X inactivation center and therefore the *XIST* gene remains inactive in mouse-human somatic cell hybrids (Brown and Willard, 1994) or in leukemia cells with X-chromosomal translocations (Rack et al., 1994). These studies suggest that *Xist* is not essential to maintain X-inactivation in differentiated cells.

However, the cells used in these studies were highly selected and might not truly represent X-inactivation in wild type cells. Indeed, *XIST* RNA does not localize correctly to the inactive X in mouse-human hybrid cells (Hansen et al., 1998). Instead, the RNA is diffuse in a much larger area of the nucleus.

We sought to address the role of *Xist* in karyotypically normal differentiated cells by creating a conditional mutant allele of the gene using the Cre-loxP system (Sauer and Henderson, 1988). We found that the loss of *Xist* in mouse female embryonic fibroblasts does not disrupt dosage compensation: in the vast majority of the cells the inactive X remains transcriptionally silent, late replicating and hypoacetylated on histone H4. However, the mutant inactive X is no longer enriched for histone macroH2A1. These results indicate that a functional *Xist* gene is necessary for preferential association of histone macroH2A1 with the inactive X; however, neither *Xist* RNA nor macroH2A1 enrichment on the inactive X are required to maintain the inactive state.

**RESULTS**

*Generation of mouse embryonic fibroblasts with a conditional *Xist* allele*

We sequentially inserted loxP sites into intron 3 and into a site located 5 kb upstream of the somatic cell promoter P1. Each targeting step was followed by Cre mediated excision of the selection cassette (Fig. 1). In the resulting *Xist^flax* allele the
inserted loxP sites flank a region of the gene that is larger than previous deletions known to inactivate the gene (Marahrens et al., 1997; Penny et al., 1996).

We generated mice and mouse embryonic fibroblasts carrying the Xist\textsuperscript{2lox} allele. Since female mice randomly inactivate one of the X chromosomes and Xist is only expressed from the inactive X, 50\% of Xist\textsuperscript{2lox} fibroblasts will transcribe the Xist\textsuperscript{2lox} allele and 50\% the wild type allele. To generate a population of cells in which the Xist\textsuperscript{2lox} allele is always on the inactive X, we bred mice carrying Xist\textsuperscript{2lox} with mice carrying a null Xist allele (Xist\textsuperscript{a}) (Marahrens et al., 1997). As the chromosome carrying Xist\textsuperscript{a} cannot be inactivated (Marahrens et al., 1998), 100\% of Xist\textsuperscript{2lox/a} cells will transcribe Xist\textsuperscript{2lox}.

Xist\textsuperscript{2lox/\gamma} cells that do not transcribe Xist were used as control.

To induce deletion of Xist\textsuperscript{2lox} in fibroblasts, we infected cells with an Adenovirus carrying the gene for Cre recombinase (Anton and Graham, 1995). The deleted allele will be referred to as Xist\textsuperscript{1lox} (Fig. 2). After Cre mediated deletion of Xist, half of Xist\textsuperscript{1lox/+} and all of Xist\textsuperscript{1lox/\alpha} cells are expected to cease expressing Xist RNA (Fig. 2a). To confirm that the Xist\textsuperscript{1lox} allele does not transcribe RNA, we performed RNA fluorescent in situ hybridization (FISH) using an Xist probe. Prior to Adenoviral infection, we detected an Xist RNA signal in 97\% of Xist\textsuperscript{2lox/+} and Xist\textsuperscript{2lox/\alpha} cells, but not in male Xist\textsuperscript{2lox/\gamma} cells. As predicted, after Cre mediated excision of Xist, Xist RNA was detected in 55\% of Xist\textsuperscript{1lox/+} cells, however, it was absent from Xist\textsuperscript{1lox/\alpha} or male Xist\textsuperscript{1lox/\gamma} cells (Fig 3a and b).

**X-linked gene expression remains monoallelic in Xist mutant fibroblasts**

Xist deficient female fibroblasts appear to proliferate normally and senesce no sooner than control cultures (data not shown). Since biallelic expression of X-linked genes is deleterious in differentiated cells (Marahrens et al., 1997), these results suggest that dosage compensation is maintained in the absence of Xist RNA expression. To determine whether X-inactivated genes indeed remain silent, we performed double labeling RNA FISH experiments using probes for two genes subject to X-inactivation, P\textit{gk-1} and H\textit{prt},
in combination with an Xist RNA probe (Fig. 3a). Prior to Cre mediated deletion of Xist, a Pglk-1 signal is detected in about 50% of nuclei and an Hprt signal in about 60%, consistent with previous studies on wild type female fibroblasts (Panning and Jaenisch, 1996; Sheardown et al., 1997). In about 5% of nuclei we detected two Pglk-1 or Hprt signals. Using DNA FISH to detect X chromosomes, and RNA FISH to detect X-linked gene expression, these nuclei were shown to be tetraploid (Fig. 3c). Tetraploid cells contain two active and two inactive X chromosomes, therefore we expect to see two Pglk-1 RNA signals from the two active X chromosomes. After Cre mediated deletion of Xist, expression of Pglk-1 and Hprt remained monoallelic (Fig. 3a) and the percentage of cells expressing X-inactivated genes did not increase (Fig. 3b), suggesting that the mutant inactive X remains transcriptionally silent.

The mutant inactive X maintains properties of heterochromatin

Delayed replication timing and underacetylation of amino terminal lysines of histone H4 are correlated with transcriptional silencing and are characteristics of the inactive X heterochromatin (Jeppesen and Turner, 1993; Keohane et al., 1996; Priest et al., 1967). To test whether the loss of Xisr affected replication timing, fibroblasts were labeled with BrdU during late S phase and BrdU incorporation was tested using a monoclonal anti-BrdU antibody on metaphase chromosomes. We detected late replicating X-chromosomes in 80% of labeled metaphases of Xisr inactive fibroblasts (Fig. 4a). In the remaining metaphase spreads all chromosomes were labeled, presumably because those cells were in mid S phase when BrdU was added.

To determine whether the loss of Xist RNA led to aberrant histone acetylation, we stained metaphases spreads with antisera against acetylated isoforms of histone H4. A weakly labeled X-chromosome was seen in all metaphase spreads of Xisr inactive cells (Fig. 4b). Therefore, we concluded that in the absence of Xist RNA, the inactive X remains heterochromatic.
The effect of long term culture

We considered the possibility that reactivation of the inactive X required more cell divisions than the limited lifespan of primary cells allowed. To test this possibility, we derived permanent $Xist^{loxa+/+}$ cell lines by SV40 T-antigen transformation. After Adenovirus-Cre mediated deletion of $Xist$, $Xist^{lox+/+}$ clones were derived from single cells and tested for Xist RNA expression. Both predicted types of subclones were isolated: clones that deleted $Xist$ from the active X, and therefore maintained $Xist$ RNA expression (called $Xist^{loxa+Xa+/+}$ clones), and clones that deleted $Xist$ from the inactive X and hence did not express $Xist$ ($Xist^{loxa+Xii+/+}$ clones). We found that in $Xist^{loxa+Xii+/+}$ clones the mutant inactive X remained late replicating, indicating that prolonged culture in the absence of Xist RNA will not reactivate the chromosome (data not shown).

Deletion of $Xist$ disrupts histone macroH2A1 localization

Histone macroH2A1 was recently shown to preferentially associate with the inactive X forming a macrochromatin body (MCB), although the protein can be found throughout the entire nucleus (Costanzi and Pehrson, 1998). The function of this histone is unknown. We tested whether histone macroH2A1 localizes to the inactive X in female cells lacking $Xist$ by immunostaining cells with macroH2A1 antisera (Costanzi and Pehrson, 1998) (Fig. 5). Though we observed nuclear staining in all cell types, macrochromatin bodies were only found in fibroblasts that expressed Xist RNA. Approximately 60-80% of nuclei of female wild type, uninfected $Xist^{loxa+}$ and $Xist^{loxa-}$ primary fibroblasts, and Xist expressing $Xist^{loxa+Xa+/+}$ SV40 T-antigen transformed clones contained detectable MCBs. However, in $Xist^{loxa+}$ primary fibroblasts, in which Xist is being transcribed in only 50% of cells, MCBs were seen in only 37% of nuclei. Moreover, we did not detect any brightly staining MCBs in nuclei that do not express Xist: $Xist^{loxa+}$ primary fibroblasts and $Xist^{loxa+Xii+/+}$ transformed clones (Fig. 5a). MCBs colocalized with
*Xist* expressing chromosomes (Fig. 5b). In cells that do not express *Xist*, neither X chromosome associated preferentially with macroH2A1 (Fig. 5c). These results suggest that histone macroH2A1 deposition to the inactive X requires *Xist* RNA, and in the absence of *Xist* RNA, the inactive X chromatin contains no more macroH2A1 than autosomal chromosomes or the active X. Our data also imply that maintenance of X-inactivation does not depend on preferential association of the inactive X with macroH2A1.

**DISCUSSION**

We generated a precise deletion of *Xist* in normal female fibroblasts. We conclude that in absence of *Xist* in the majority of the cells the mutant inactive X remains transcriptionally silent, late replicating and underacetylated on histone H4. Since dosage compensation is crucial for the proper functioning of the cell, it is likely that multiple independent mechanisms cooperate to keep the inactive X silent, and removal of any one of these is not sufficient for reactivation. Our results indicate that late replication and histone hypoacetylation are not dependent on *Xist* for maintenance and that they are sufficient (in cooperation with other silencing mechanisms) to maintain X-inactivation in the absence of *Xist* RNA. However, since the assays used in this study are not sensitive enough to detect reactivation in a small percentage of cells, we cannot exclude the possibility that X-inactivation in the absence of *Xist* is less stable. Other, more sensitive assays are needed to address this question.

Although the inactive X remains inactive in the absence of *Xist*, our results indicate that it is no longer enriched for macroH2A1. These observations suggest a role for *Xist* RNA in remodeling the chromatin of the inactive X, shedding new light on its role in X-inactivation and its maintenance. *Xist* RNA and histone macroH2A1 may directly interact, as suggested earlier on the basis of sequence homology between the nonhistone region of macroH2A1 and a conserved domain with a potential RNA binding activity (Pehrson and Fuji, 1998). Alternatively, *Xist* RNA may mediate macroH2A1 localization indirectly by
altering the structure of the inactive X in some way to allow access of macroH2A1 to the chromatin. Xist RNA interacts with the nuclear matrix and remains localized there even after extraction of chromosomal DNA and histones from the nucleus (Clemson et al., 1996). MacroH2A1, on the other hand, is tightly bound to the nucleosome (Pehrson and Fried, 1992). Therefore an Xist RNA-macroH2A1 interaction may represent a link between the chromatin of the inactive X and the nuclear matrix.

MATERIALS AND METHODS

Targeting in ES cells and generation of mice. Targeting vector pX3lox was constructed from plasmid pX3 containing a genomic fragment from intron 1 to the middle of exon 7 in Bluescript vector. Targeting vector pXprlox was constructed from plasmid pP1prom, containing a 10 kb XhoI fragment from 9 kb upstream of promoter P1 to 900 bp into exon 1. The positive-negative selection cassette was constructed from pBS246 containing two loxP sites flanking a polylinker, and tgCMV/HyTK that contains a hygromycin resistance-thymidine kinase fusion gene under the CMV promoter. To prepare the loxP-CMVhygroTK-loxP cassette, a 3 kb NotI fragment of tgCMV/HyTK was ligated into an EcoRV linearized pBS246, after which the cassette was released by NotI digestion. To construct pX3lox, the selection cassette was inserted into pX3 that had been linearized with a NdeI partial digest. To construct pXprlox, the selection cassette was ligated into pP1prom that had been linearized with a partial BglII digest.

Embryonic stem cell (ES cell) culture has been described before (Li et al., 1992). When appropriate, hygromycin (to 150 µg/mL final concentration) or gancyclovir (2 µg/mL final) was added to the media. The wild type male ES cell line J1 was electroporated linearized pX3lox, and hygromycin resistant colonies were selected, picked, and analyzed by Southern blotting of NcoI digested DNA and using probe 4. Approximately 60% of the picked clones contained a correctly targeted Xist allele with loxP-CMVhygroTK-loxP inserted into intron 3 (inthyTK allele). To remove the hygroTK

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cassette, supercoiled pOG231 plasmid (CMV promoter-synthetic intron-Cre coding sequence) was electroporated into targeted ES cells. Gancyclovir resistant colonies were picked and analyzed by PCR using primers Xint3F and Xint3R. Over 70% of the picked colonies correctly excised the hygroTK cassette, leaving a single loxP site behind (intloxl allele). These ES cells were then electroporated with linearized pXprlox, and hygromycin resistant colonies were again selected, picked and analyzed by Southern blot analysis of XbaI digested DNA using probe 6. Targeting frequency was 15%. Targeted clones (3loxl allele) were then electroporated with supercoiled pOG231, gancyclovir resistant colonies were analyzed by Southern blotting of XbaI digested DNA and using probes 6 and 7. About 50% of the colonies excised the hygroTK cassette only (2loxl allele) and 20% excised the entire region from upstream of the promoter to intron 3 (1loxl allele). Chimeric mice and germline pups were obtained from two independently derived Xisr<sup>2loxl</sup> ES cell clones.

Probe 4 is a 2 kb XbaI-BglII fragment at the end of exon 7; probe 6 is a 2 kb XhoI-XbaI fragment at the beginning of exon 1; probe 7 is a 1.1 kb EcoRI-XbaI fragment at the beginning of exon 7. Primer Xint3F sequence is: 5'-GGCCAGTTTCGTGACACCCCTA-3'; Xint3R: 5'-CACTGGCAAGGTGAATAGCA-3'.

**Mouse embryonic fibroblast culture and Adenoviral infection.** Primary mouse embryonic fibroblasts (MEFs) were derived from E14 embryos by dissociation and trypsinization, cultured and when necessary immortalized with SV40 T-antigen (Jat et al., 1986). For Cre mediated recombination in fibroblasts, cells were infected with an Adenovirus vector carrying the gene for Cre recombinase (Anton and Graham, 1995). Infection was carried out in monolayer culture in DMEM/2% fetal calf serum for two hours. The lowest possible multiplicity of infection that yielded 100% recombination without cytotoxic effects was experimentally determined. After infection, cells were grown in DMEM/15% fetal calf serum with antibiotics. The efficiency of Cre mediated recombination was analyzed by Southern blotting of XbaI digested DNA and using probe
7. Single cell-derived clones were isolated from limiting dilution of SV40 T-antigen immortalized, Adenovirus-Cre infected culture.

**Cell fixation and FISH.** For fluorescent in situ hybridization, cells were grown in multichamber slides, fixed in 4% paraformaldehyde, and stored in 70% ethanol. Hybridization, washing and detection of probes have been described before (Panning and Jaenisch, 1996). A probe for Xist RNA was generated from pXEXTAP, a plasmid containing a genomic fragment from intron 1 to the end of exon 7. The P\textit{gk-1} probe was generated from pCAB17, 17 kb genomic clone, and the \textit{Hprt} probe from pRV18.6, an 18.6 kb genomic clone. Probes were generated using either nick translation with Cy3-dCTP (Amersham) or random priming with biotinylated dCTP (GIBCO). X-chromosomes were detected using a biotinylated X-chromosome paint (Oncor). When necessary, the X-chromosome paint signal was amplified after FITC-avidin detection, using a biotinylated-anti-avidin antibody (Vector) followed by another round of detection with FITC-avidin.

**Analysis of DNA replication timing.** Cells were grown in the presence of 30 \textmu M BrdU for the last 4.5-5 hours prior to fixation. Colcemid was added for the last 2 hours. Cells were fixed in methanol/acetic acid (3:1) and dropped onto slides. Slides were denatured in 70% formamide/2X SSC at 70-74°C for 2 minutes. BrdU signal was detected using a monoclonal anti-BrdU antibody (Becton Dickinson) and Texas red anti-mouse antibody (Vector). Immediately after detection of incorporated BrdU, slides were dehydrated and incubated with X-chromosome paint probe (Oncor) without further denaturation of the chromosomes.

**Analysis of histone H4 acetylation.** For labeling chromosomes with antisera against acetylated histones, cells were trypsinized, washed in PBS, swollen in hypotonic solution (75 mM KCl) and dropped onto slides. Antibody staining of unfixed chromosomes and postfixation were performed as described (Jeppesen and Turner, 1993). The antibodies used were R17, recognizing histone H4 acetylated at all four amino terminal lysines, and R41/5 recognizing histone H4 acetylated at lysine 5 (Jeppesen and Turner, 1993) (Serotec).
Identical results were obtained with both. To detect X chromosomes after immunostaining, slides were denatured in 70% formamide/2X SSC at 70-74°C for 10 minutes, dehydrate and incubated with X chromosome paint probe (Oncor).

**Histone macroH2A1 immunofluorescence.** Cells were grown on multichamber slides, and fixed in 4% paraformaldehyde. Slides were incubated for 30 minutes in blocking buffer (1x PBS, 5% goat serum, 0.2% Tween, 0.2% fish skin gelatin), followed by incubation with the primary anti-macroH2A1 antibody (Costanzi and Pehrson, 1998) in blocking buffer for 2 hours at room temperature. Slides were then washed three times in PBS/0.2% Tween for 5 minutes each, incubated with the secondary antibody (fluorescein or Texas red anti-rabbit (Vector)) in blocking buffer for 30 minutes at room temperature, washed again in PBS/Tween, and counterstained with DAPI. For combined RNA FISH/immunofluorescence, freshly prepared slides were incubated with biotinylated Xist RNA FISH probe, the signal detected with fluorescein-avidin and crosslinked with 4% paraformaldehyde. The slides were then used for immunostaining as above. For combined DNA FISH/immunofluorescence, immunofluorescence for performed as above, the antibody was crosslinked in 4% paraformaldehyde, the slides were denatured in 70% formamide/2X SSC at 70-74°C for 10 minutes and DNA FISH was performed using an X chromosome paint probe. Fluorescent images were captured on Kodak Ektachrome 1600 slide film using a Zeiss Axioskop. Slides were converted to digital images using Kodak Sprintscan 35 + scanner and color channels were merged in Photoshop 5.0.

**ACKNOWLEDGEMENTS**

We would like to thank Steven O’Gormann for the pOG231 plasmid, Jessie Dausmann for blastocyst injections, Ruth Curry for help with maintaining the mouse colony, Anton Wutz, Sara Cherry and Ted Rasmussen for critical reading of the manuscript and discussions. This work was supported by a grant to R.J. from the National Institute of Health/National Cancer Institute (R35-CA44339).
REFERENCES


Figure 1. Generating the conditional Xist allele. (A) The strategy used to target intron 3 in step (I), and upstream of the promoter in step (III), and to remove the selectable markers in steps II and IV. Targeting vectors pXprlox and pX3lox, and the various Xist alleles created in the process are also shown. (B) Southern blot of NcoI digested DNA hybridized with probe 4 showing correct targeting of intron 3, creating the intthTK allele. (C) PCR assay using primers flanking the insertion site in intron 3, showing correct Cre mediated excision of the selectable marker, creating the intlox allele. (D) Southern blot of XbaI digested DNA hybridized with probe 6 showing correct targeting upstream, generating the 3lox allele. (E) Southern blot of XbaI digested DNA hybridized with probes 6 and 7, showing Cre mediated recombination that removes either the selectable marker only (2lox allele) or the entire region between upstream of the promoter and intron 3 (1lox allele).

X: XbaI sites; N: NcoI sites; pr4, pr6, pr7: probes 4, 6, and 7, respectively.
Figure 2. Fibroblast genotypes. (A) Diagram of cell types and their X chromosomal content with Xist genotypes prior to and following Cre mediated recombination. (B) Cre mediated recombination in Xist2lox/+, Xist2lox/Δ, and male Xist2lox/Y cells, generating fibroblasts with genotype Xist1lox/+, Xist1lox/Δ, and Xist1lox/Y. Southern blot of XbaI digested DNA using probe 7 shows complete recombination in fibroblasts four days after infection with Adenovirus-Cre. Note that the wild type and the Xist2lox/+ alleles comigrate on this Southern. These two alleles can be distinguished by PCR.
**Figure 3.** Xist RNA is absent and X-linked genes *Pgk-1* and *Hprt* are monoallelically expressed in mutant fibroblasts. (A) RNA FISH using Cy3 labeled Xist probe (red) and biotin labeled *Pgk-1* probe (green) on *Xistlox/+* and *Xistlox/Δ* cells. Xist RNA is expressed in half of *Xistlox/+*, but is absent in *Xistlox/Δ* cells. A single locus of *Pgk-1* transcription in every nucleus is indicated by arrows. (B) Percentage of cells expressing Xist, *Pgk-1* and *Hprt*. A similar percentage of cells expressed *Pgk-1* and *Hprt* before and after Cre mediated deletion of Xist. Results shown were obtained 9 days postinfection. Similar results were obtained 4 or 17 days following Cre mediated recombination. Among *Xistlox/+* fibroblasts, *Pgk-1* and *Hprt* signals were counted separately in Xist expressing cells from those that did not express Xist. (n>200) (C) Cells with two *Pgk-1* signals are tetraploid. RNA FISH to detect *Pgk-1* transcription (green) was followed by DNA FISH to detect X chromosomes (red). The 5-7% of cells that contained two *Pgk-1* signals had four X chromosomes indicating that they are tetraploid with active and two inactive X chromosomes.

*In these cultures, very few cells (2%) were found to have an Xist signal. These exceptional cells presumably escaped Adenoviral infection and were excluded from counting of *Pgk-1* and *Hprt* signals.
Figure 4. The mutant inactive X remains late replicating and hypoacetylated in Xist\textsubscript{lox}/+ cells. (A) The mutant inactive X is late replicating. Late replicating X chromosomes were detected by labeling cells with BrdU in late S phase followed by immunostaining with monoclonal anti-BrdU antibody (red) on metaphase chromosomes (DAPI, blue). X chromosomes were identified using X chromosome paint probe (green) on the same metaphase spread. A single X is late replicating. The experiment was performed 9 days postinfection. (B) The mutant inactive X is hypoacetylated on histone H4. Indirect immunofluorescence using antiserum raised against acetylated histone H4 (red). X chromosomes were identified with X chromosome paint probe (green). A single X chromosome is staining pale with the antibody. High contrast DAPI image of the chromosomes is shown in grayscale on the right. Immunofluorescence was performed 9 days following Adenovirus-Cre infection.
Figure 5. Xist RNA is necessary for enrichment of the inactive X in histone macroH2A1. (A) Immunofluorescence using anti-histone macroH2A1 antiserum (green) on Xist^{loxa+}, Xist^{loxaΔ} primary fibroblasts and on Xist^{loxa}/+ and Xist^{loxi}/+ transformed fibroblast clone nuclei (DAPI, blue). MCBs are only seen in cultures that also transcribe Xist RNA. (B) Colocalization of Xist RNA and MCBs. Xist RNA FISH (green) followed by immunostaining with histone macroH2A1 antiserum (red), demonstrating that histone macroH2A1 concentrates only on inactive X chromosomes that also transcribe Xist RNA. (C) In cells that do not express Xist, neither X is enriched in histone macroH2A1. Xist^{loxaΔ} cells were stained with histone macroH2A1 antiserum (red), denatured and hybridized with X chromosome paint probe (green).
Chapter 3.

Synergism of Xist RNA, DNA methylation and histone hypoacetylation in maintaining X chromosome inactivation

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Data presented in this chapter have been published in J Cell Biol (2001) 153:773-84

Respective contributions: András Nagy provided the GFP-transgenic mice.

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ABSTRACT

Xist RNA expression, methylation of CpG islands, and hypoacetylation of histone H4 are distinguishing features of inactive X chromatin. Here we show that these silencing mechanisms act synergistically to maintain the inactive state. Xist RNA has been shown to be essential for initiation of X-inactivation, but not required for maintenance of the inactive state. We have developed a system in which the reactivation frequency of individual X-linked genes can be assessed quantitatively. Using a conditional mutant Xist allele, we provide direct evidence that loss of Xist RNA destabilizes the inactive state in somatic cells, leading to an increased reactivation frequency of an X-linked GFP transgene and of the endogenous Hprt gene in mouse embryonic fibroblasts. Demethylation of DNA, using 5-azadC or by introducing a mutation in Dnmt1, and inhibition of histone hypoacetylation using TSA further increases reactivation in Xist mutant fibroblasts, indicating a synergistic interaction of X chromosome silencing mechanisms.

INTRODUCTION

In mammals equal X-linked gene dosage between the sexes is achieved by X chromosome inactivation in females. The inactivated X chromosome resembles constitutive heterochromatin in that it is condensed in interphase (Barr, 1962), hypoacetylated on histone H4 (Jeppesen and Turner, 1993), and replicates late in S phase (Priest et al., 1967). It is also methylated on CpG islands of housekeeping genes (Norris et al., 1991) and is enriched in histone macroH2A1, a histone H2A variant with a large nonhistone domain (Costanzi and Pehrson, 1998). The inactive X (Xi) expresses Xist (Brown et al., 1991; Borsani et al., 1991; Brockdorff et al., 1991), a nuclear, untranslated RNA (Brockdorff et al., 1992) that coats the chromosome in cis (Clemson et al., 1996). Both X chromosomes of an undifferentiated embryonic female cell are active, and X inactivation is initiated at the time of differentiation in vitro or in vivo (Monk and Harper, 1979).
Once an X chromosome is inactivated in a cell, the inactive state of the chromosome is clonally inherited through many rounds of cell division.

The most remarkable feature of Xi chromatin is its stability with respect to reactivation. Overall experimental reactivation of the entire chromosome in somatic cells has not been observed. Reactivation of one or few genes on the Xi has been seen, but the rate of reactivation is low, on the order of $10^3$ to $10^4$, in cultured somatic cells (Graves, 1982; Mohandas et al., 1981). Many lines of evidence indicate that multiple molecular mechanisms are responsible for the high fidelity of maintenance of X-inactivation (reviewed in Migeon, 1994), but the contribution of individual mechanisms to silencing and their complex relationship remains to be elucidated.

Methylation of CpG dinucleotides in the promoter region of repressed genes has long been thought of as a mechanism stabilizing X chromosome inactivation. 5-azadC, an inhibitor of DNA methyltransferase 1 (Dnmt1), the major enzyme responsible for maintaining genomic methylation patterns, has been used to derepress several Xi-linked genes, providing the experimental evidence for the importance of methylation in X-inactivation (Graves, 1982; Mohandas et al., 1981). Methylation of the CpG island of Hprt follows X-inactivation by several days, implying that methylation plays a maintenance role (Lock et al., 1987). An in vivo demonstration of the importance of CpG methylation in X-inactivation maintenance is the instability of silencing on the Xi of ICF (immunodeficiency, centromeric instability, facial anomalies) patients. The Xi of patients suffering from ICF syndrome is hypomethylated at all CpG islands analyzed (Hansen et al., 2000). The syndrome is caused by a defect in the DNA methyltransferase DNMT3B (Hansen et al., 1999, Okano et al., 1999, Xu et al., 1999), suggesting that the enzyme may be responsible for establishing CpG methylation on Xi. X-inactivation in the virtual absence of CpG methylation is less stable, as evidenced by reactivation of some loci in cells from these patients (Hansen et al., 2000).

Underacetylation of amino terminal lysine residues on histones H3 and H4 is another feature of Xi chromatin (Jeppesen and Turner, 1993). It is well established that transcriptional activity of genes is regulated by histone acetylation. Histone deacetylation generally correlates with
transcriptional silencing, and high levels of acetylation with transcriptional activity (Cheung et al., 2000). However, reactivation of X-inactivated genes by altering histone acetylation levels has not been reported. The appearance of a hypoacetylated X chromosome is a late event during differentiation and X-inactivation, suggesting that deacetylation of histones is a maintenance rather than establishment mechanism (Keohane et al., 1996).

Xist RNA is essential for the initiation of X chromosome inactivation in cis (Marahrens et al., 1997; Penny et al., 1996). However, several lines of evidence indicate that after X inactivation has been established, Xist is no longer required for maintenance. In studies using mouse-human somatic cell hybrids a human Xi chromosome fragment that lacked the XIST gene remained transcriptionally silent and its sensitivity to reactivation by 5-azadC did not increase (Brown and Willard, 1994). Similarly, in human leukemia cells an Xi derived isodicentric chromosome maintained its inactive state despite missing the XIST gene (Rack et al., 1994). To directly address the role of continued Xist RNA expression in karyotypically normal somatic cells, we have previously generated a conditional Xist allele (Csankovszki et al., 1999) using the Cre-loxP system (Sauer and Henderson, 1988). After Cre mediated deletion of Xist in primary mouse embryonic fibroblasts, the Xi remained silent (Csankovszki et al., 1999), again arguing that in the absence of Xist RNA, other silencing mechanisms are sufficient to keep Xi silent. Another study used embryonic stem cells expressing an inducible Xist cDNA transgene, in which the timing of expression can be experimentally manipulated (Wutz and Jaenisch, 2000). This approach defined an initial differentiation time window in which X inactivation is reversible and Xist dependent, followed by irreversible and Xist independent X-inactivation in fully differentiated cells (Wutz and Jaenisch, 2000).

Nevertheless, continued transcription of Xist RNA and its close association with Xi throughout the lifetime of the female mammal suggests a role for Xist in somatic cells (Clemson et al., 1996). Indeed, in rodent-human somatic cell hybrids where the human XIST RNA does not localize correctly to Xi (Clemson et al., 1998; Hansen et al., 1998), the stability of silencing is greatly reduced. It has been speculated that reduced efficiency of silencing in hybrid cells is due to
the absence of correctly functioning XIST RNA (Clemson et al., 1998; Hansen et al., 1998). Furthermore, deletion of Xist in mouse embryonic fibroblasts disrupts preferential localization of histone macroH2A1 to Xi, and this alteration of chromatin may also lead to decreased stability of silencing (Csankovszki et al., 1999).

In order to assess the possible role of Xist RNA in X-inactivation maintenance and to study the relative contribution of Xist, DNA methylation and histone hypoacetylation, we developed a system in which even low levels of reactivation can be quantitatively measured. Reactivation of two markers on Xi were studied, a GFP (green fluorescent protein) transgene (Hadjantonakis et al., 1998) and the endogenous Hprt (hypoxanthine phosphoribosyl transferase) gene. Using a conditional deletion of Xist (Csankovszki et al., 1999), we provide direct evidence for the first time that Xist RNA contributes to silencing in somatic cells. Additionally, using the drugs 5-azadC and Trichostatin A (TSA) and by introducing a mutation in the DNA methyltransferase 1 (Dnmt1) gene (Jackson-Grusby et al., 2001), we show that Xist RNA, DNA methylation and histone hypoacetylation act synergistically to achieve a highly stable inactive state.

RESULTS

Generation of Xist mutant mouse embryonic fibroblasts

In order to study the stability of silencing on Xi, we generated fibroblasts with two Xi-linked markers, where reactivation was designed to be detectable even at low frequencies. One marker was an X-linked GFP transgene that is subject to X-inactivation (Hadjantonakis et al., 1998). When the GFP transgene was located on Xi, cells were GFP negative and reactivation could be monitored using FACS analysis. The insertion site of the transgene was determined by DNA FISH (fluorescent in situ hybridization) and GFP was mapped to a position near the centromere (Fig. 1A and 5A). The second marker was the endogenous X-linked Hprt gene, the activity of which is required in order to survive in HAT containing medium. When cells carrying a wild type Hprt allele on Xi and a mutant allele, Hprt<sup>a</sup> (Hooper et al., 1987), on the active X (Xa) are subjected to HAT selection, even a few reactivants in a large population can be isolated. To
generate a homogeneous population of cells with defined active and inactive X chromosomes, a
null mutation in the Xist gene, Xist<sup>a</sup> (Marahrens et al., 1997), was introduced onto the
chromosome carrying Hprt<sup>a</sup>. Since the Xist<sup>a</sup> allele cannot be chosen for X-inactivation (Marahrens
et al., 1998), cells with genotype Hprt<sup>a</sup>; GFP/Xist<sup>a</sup>; Hprt<sup>a</sup> carry the GFP transgene and the Hprt<sup>a</sup>
allele on Xi.

To study the effect of deletion of Xist from Xi, a conditional allele of the gene, Xist<sup>lox</sup>
(Csankovszki et al., 1999) was introduced onto the chromosome. Cells with genotype Xist<sup>lox</sup>;
Hprt<sup>a</sup>; GFP/Xist<sup>a</sup>; Hprt<sup>a</sup> will be referred to as conditional mutants. Control cells with genotype
Xist<sup>a</sup>; Hprt<sup>a</sup>; GFP/Xist<sup>a</sup>; Hprt<sup>a</sup> lack the Xist<sup>lox</sup> allele, and hence cannot delete the Xist gene (Fig.
1B). To induce Cre mediated deletion of Xist<sup>lox</sup>, we infected cells with an Adenovirus carrying
the gene encoding Cre recombinase (Anton and Graham, 1995). An Xist<sup>lox</sup> to Xist<sup>lox</sup>
recombination was observed in 100% of the cells, as assayed by Southern blotting (Fig. 1C).
Conditional mutants and controls were treated identically and infections and all further analyses
were done in triplicates. Cre mediated deletion of Xist took place over a period of 2-3 days and
Xist RNA levels were undetectable by day 4 [(Csankovszki et al., 1999) and data not shown]. In
a previous study we showed that deletion of Xist in mouse embryonic fibroblasts does not interfere
with late replication timing and underacetylation of histone H4 residues, but it disrupts preferential
localization of histone macroH2A1 to Xi (Csankovszki et al., 1999).

**Deletion of Xist leads to increased reactivation of GFP and Hprt**

GFP expression was analyzed using FACS in Xist mutant and control cells that were infected with
Adenovirus-Cre or left untreated. GFP fluorescence was compared to autofluorescence and cells
showing greater GFP fluorescence than autofluorescence were counted as positive. We found that
the number of cells expressing GFP was dependent on cell density and both the number of cells
expressing GFP and the intensity of fluorescence decreased upon long term culture (data not
shown). Therefore, care was taken to ensure that an equal number of cells were plated for each
sample and cells from the same early passage were used for each experiment. Results of a typical
experiment are shown in Fig. 2. Fibroblasts not containing the GFP transgene (genotype +/Y) were negative, while over 99.9% of cells with GFP on Xa (GFP/Y) were positive. The majority of cells with GFP on Xi were also GFP negative with a small number of positives, about 10-20 in 100,000 representing the spontaneous reactivation frequency (Fig. 2A). In primary Xist conditional mutant fibroblasts after Cre mediated deletion of Xist, the number of GFP positive cells increased about 2-fold (Fig. 2B). This increase cannot be attributed to the effect of Adenovirus infection, as the number of GFP positive cells did not increase in control cells after viral infection. Although the effect is small, we consider it significant as we consistently detected a two-fold difference in GFP expressing cells between mutant and control cells in five independent repetitions of the assay.

To test whether long term culture leads to further decrease in the stability of silencing and thus to an increase in GFP reactivation frequency, we derived permanent cell lines by SV40 T-antigen transformation and cultured the cells for over 2 months. GFP expression was analyzed at various timepoints (Fig. 2C). Seven days after Adenoviral infection, the transformed cells behaved similarly to primary cells: the conditional mutants exhibited a 2-3 fold increase in the number of GFP positive cells after Cre mediated deletion of Xist, while there was no difference between infected and uninfected control cells. However, by day 14 postinfection, the difference between the infected and uninfected mutant cells disappeared, and the proportion of GFP positive cells remained unchanged for over two months. Reactivated GFP alleles are likely subject to resilencing, consistent with our observations that GFP expression declines over time and with earlier studies that showed that this transgene is subject to nonspecific silencing, even when carried on Xa (Eggan et al., 2000). In addition, it is possible that reactivation of X-inactivated genes confers selective disadvantage on the cells leading to a decrease in the number of GFP positive cells over time (see section on clones with reactivated genes).

We next analyzed reactivation of an endogenous X-inactivated gene, Hprt, by subjecting cells to selection in HAT medium. Since HAT resistant colonies from rare reactivants can only be obtained in permanent cell lines, these experiments were performed in SV40 T-antigen
immortalized cells. Two million cells were plated and selected in HAT medium for two weeks, after which plates were fixed and stained to count the number of colonies (Fig. 3A). Very few (<1/plate) colonies were seen on control plates and on plates containing conditional mutant fibroblasts prior to Cre mediated Xist, indicating that the spontaneous reactivation frequency of Hprt is very low. However, after deletion of Xist, a small but significant number of cells reactivated Hprt, and Xist mutant cultures yielded about 30-70 HAT resistant colonies per plate. The proportion of HAT resistant cell in the population increased for the first four weeks in culture indicating ongoing reactivation of Hprt. Furthermore, HAT resistant cells were detected even after 3 months in culture (Fig. 3B). Northern analysis of total RNA indicated that different clones transcribed different amounts of Hprt RNA (data not shown). We conclude that deletion of Xist leads to an increased albeit low frequency of reactivation of an endogenous X-inactivated gene, Hprt, indicating that Xist RNA contributes to stabilizing the inactive state in the maintenance phase of X-inactivation.

Patchy reactivation of X-inactivated genes in Xist mutant fibroblasts

As the nature of Xist RNA mediated silencing has not been studied in detail in somatic cells, we next examined whether Xist RNA coordinately silences genes on Xi or whether genes are regulated independently of one another. First, we isolated HAT resistant clones with a reactivated Hprt allele, and asked whether these cells are also GFP positive (Fig. 4). We analyzed two types of clones, fast growing ones that grew almost as well as the unselected population (n=6), and slow growing ones that divided barely enough to yield sufficient cells for FACS analysis (n=7). In Xist mutant and Xist wild type bulk populations prior to HAT selection, the number of GFP positive cells was about 1 in 10,000. Among fast growing clones the variation was large, but on average the clones contained 10-20 times more GFP positive cells than the unselected populations. In slow growing clones the number of GFP positive cells was on average another 10-fold higher (Fig. 4). Yet, even in the clone with the highest proportion of GFP positive cells, over 98% of cells remained GFP negative. Therefore, most cells that reactivated Hprt generally did not also
reactivate GFP. However, a chromosome that reactivated Hprt was more prone to reactivate GFP than a chromosome with an inactive Hprt gene.

It is interesting to note that HAT resistant clones with a higher proportion of GFP positive cells grew slower than those with fewer GFP positive cells. This result is consistent with the conclusion that reactivation of X-inactivated genes confers selective disadvantage on the cells. In clones with a higher proportion of GFP positive cells, the more extensive reactivation is more detrimental to cell growth.

Finally, we isolated GFP positive clones by sorting and expanding individual GFP positive cells, and tested whether these clones also reactivated Hprt. Similarly to the HAT resistant clones, GFP positive clones did not grow well, and most died under HAT selection without yielding a single colony. However, occasionally (2 of the 42 clones analyzed), the clone grew well in HAT containing medium, indicating that most, if not all cells of the clone were also Hprt positive. We conclude that most clones that reactivated one X-linked gene do not also reactivate another. Yet, there is a certain level of cooperativity in reactivation, as a chromosome that reactivated one gene on Xi, is more likely to reactivate another one than a chromosome that has not reactivated any gene.

**X chromosomes with reactivated genes remain late replicating**

An inactive X chromosome after deletion of Xist remains late replicating when analyzed in bulk population with presumably very few of the cells containing reactivated genes (Csankovszki et al., 1999). We wished to see whether in the reactivated clones, Xi, or a cytologically visible portion of it, became early replicating. We analyzed two slow growing HAT resistant clones with a high proportion of GFP positive cells and one GFP positive clone that was also HAT resistant. As the rest of the clones grew too poorly to obtain sufficient numbers of cells for the assay, we pooled small HAT resistant colonies and GFP positive clones and the assay was also performed on the pools. BrdU incorporation into late replicating regions was detected using an anti-BrdU antibody and Xi was identified by DNA FISH using a probe that detects the GFP transgene. In all
clones and pools analyzed, the Xi marked by the GFP transgene was late replicating (Fig. 5B and C). These results indicate that even after reactivating one or more genes on Xi, the chromosome as a whole remained inactive. We conclude that reactivation of inactive X-linked genes after loss of Xist RNA occurs at one or a few loci and does not result in a detectable change in late replication of the chromosome.

Synergism of Xist RNA, DNA methylation and histone hypoacylation in X chromosome silencing

We next examined the relationship between Xist RNA mediated silencing and inactivation by other mechanisms. DNA methylation and hypoacylation of core histones are believed to contribute to inactivation of X-linked genes (Cedar, 1988; Keohane et al., 1998). A low frequency of reactivation of X-linked genes has been observed after demethylating cells using 5-azadC (Graves, 1982; Mohandas et al., 1981). Transcriptional activation of silenced genes has also been seen after treating cells with Trichostatin A (TSA), a potent inhibitor of histone deacetylases (Yoshida et al., 1995). We wanted to see whether the Xist mutant cells with their already compromised ability to silence Xi are more sensitive to demethylation and/or inhibition of histone deacetylation than wild type cells leading to a further increase in the number of cells reactivating GFP and Hprt.

Adenovirus-Cre infected and uninfected Xist conditional mutant cells were treated with 5-azadC and/or TSA and the number of cells that reactivated GFP was analyzed using FACS (Fig. 6A). Due to the toxicity of the drugs, the experiments were performed in SV40 T-antigen transformed cells. The cells were infected with Adenovirus-Cre, allowed to recover, then treated twice with 5-azadC on days 7 and 9 postinfection. After allowing the cells to go through several rounds of cell division to achieve demethylation of DNA, half of the cultures were treated with TSA on day 12 postinfection. FACS analysis was performed on day 13. By day 13 postinfection, the Xist deletion induced increase in the number of GFP reactivants disappeared, and we could no longer observe a difference between Xist wild type and Xist deficient cells without drug treatment
(see Fig. 2C). Inhibition of histone deacetylases by itself had no effect on the number of GFP positive cells whether or not Xist was deleted. 5-azadC induced demethylation increased the number of GFP positive cells by about 20-fold in cells that did not delete Xist. However, the combined effect of Xist deletion and 5-azadC treatment was a 30-40 fold increase in GFP reactivants. 5-azadC treatment followed by TSA further increased the number of GFP positive cells by another twofold in both Xist mutant and control cells. These results are summarized in Table I. The up to 60-fold increase in the number of cells reactivating GFP indicate that the effects of Xist RNA deletion, 5-azadC and TSA treatments are synergistic, rather than simply additive.

5-azadC treatment leads to limited genomic demethylation (Fig. 7A). More extensive demethylation can be achieved by deleting Dnmt1. To study how X-inactivation is maintained in the absence of Xist RNA and DNA methylation, we bred a Dnmt1 conditional allele (Jackson-Grusby et al., 2001) into the Xist mutant colony and generated double conditional mutant fibroblasts. Fibroblast genotypes were: Xistlox/lox, GFP/Xistlox, Dnmt1lox/lox for Xist conditional mutants, and Xist−; GFP/Xistlox, Dnmt1lox/lox for control. The Dnmt1lox allele is a constitutive null mutation of the gene (Lei et al., 1996). Upon Adenovirus-Cre infection of these cells, both Xist and Dnmt1 were deleted in Xist mutant cells, while only Dnmt1 was deleted in the controls (Fig. 7B). Deletion of Dnmt1 led to a more pronounced demethylation of bulk genomic DNA than 5-azadC treatment (Fig. 7). Primary Dnmt1 mutant fibroblasts arrested within a week of Adenovirus-Cre infection, while SV40 T-antigen transformed cells continued dividing, although at a much slower rate than wild type cells (Jackson-Grusby et al., 2001). We analyzed both primary and transformed cells 7 days postinfection. A much higher proportion of cells reactivated GFP in the Dnmt1 mutants than in 5-azadC treated cells (Fig. 6B) most likely as a result of the Dnmt1 mutants being much more demethylated. Transformed cells consistently yielded more GFP reactivants possibly due to their increased ability to proliferate and further demethylate or as a result of decreased stability of silencing in transformed cells. Xist/Dnmt1 double mutants reactivated GFP in about twice as many cells as Dnmt1 single mutants, indicating again that Xist and DNA methylation cooperate to silence GFP on Xi. GFP was reactivated in up to 30% of Dnmt1/Xist
double mutant cells, representing an almost 3,000 fold increase over controls (Table I). Interestingly, the Dnmt1 mutation or even limited demethylation using 5-azadC has a much more significant effect on the number of GFP reactivants than the Xist mutation, arguing that at least for the X-linked GFP transgene, DNA methylation is a more important contributor to silencing than Xist.

To study the combined effect of demethylation and Xist deletion on an endogenous X-inactivated gene, we analyzed Hprt reactivation in Adenovirus-Cre infected or uninfected Xist conditional mutant cells, with or without 5-azadC treatment (Fig. 6C). In Adenovirus-Cre infected (Xist deleted) cultures without drug treatment we observed an about 100-fold enrichment for HAT resistant cells over uninfected (Xist wild type) cells. 5-azadC treatment of Xist wild type cells resulted in a less significant 10-fold increase in the number colonies per plate. However, 5-azadC treatment of Xist mutant cells resulted in a 50-fold increase in HAT resistant cells over untreated Xist mutant cells and an almost 5000-fold increase over untreated Xist wild type cells (Table I). The combined effect of Xist deletion and demethylation is more significant than either treatment alone, implying a synergistic interaction of Xist RNA and DNA methylation in keeping Hprt silent. It is interesting to note, that while the absolute numbers of GFP and Hprt positive cells are different, 5-azadC treatment led to comparable enrichment in reactivants for both genes (19 and 12 fold respectively). However, Xist RNA seems to play a more major role in Hprt silencing than in GFP silencing (100 fold enrichment compared to 2-3 fold). The GFP transgene may not be subject to Xist RNA mediated silencing the same way endogenous X-linked genes are regulated.

**Rate of Hprt reactivation in Xist deficient fibroblasts**

To test whether the observed HAT resistant colonies are the result of independent Hprt reactivation events or proliferation of a few reactivants, we calculated Hprt reactivation rates using the Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943). A large number of cultures of Adenovirus-Cre infected and uninfected cells were expanded from a few cells (generally <100). During expansion half of the cultures were treated with 5-azadC. After the desired culture size was
reached, cells were selected using HAT and the number of cultures that yielded HAT resistant colonies and the reactivation rates were determined (Table II). The positive cultures ranged from containing one clone to confluent plates, indicating that the reactivation event took place at different times during cultivation. The spontaneous reactivation rate of <i>Hprt</i> was as low as previously reported mutation rates in wild type cells ($10^{-9}$) (Chen et al., 1998) demonstrating the remarkably stability of X chromosome silencing. The reactivation rate increased by about 160-fold after deletion of <i>Xist</i> and 60-fold after 5-azadC treatment, indicating that both <i>Xist</i> RNA and DNA methylation contribute significantly to silencing. However, combining <i>Xist</i> deletion with 5-azadC treatment resulted in an almost 10,000-fold increase, confirming synergism of DNA methylation and <i>Xist</i> expression in the maintenance of the inactive state.

**DISCUSSION**

We generated fibroblasts in which reactivation of two genes on the X chromosome, a <i>GFP</i> transgene and the endogenous <i>Hprt</i> gene, can be detected at a low frequency. We studied the effect of loss of <i>Xist</i> RNA, demethylation of genomic DNA and inhibition of histone deacetylation, on the maintenance of X inactivation. We observed that deletion of <i>Xist</i> leads to reactivation of <i>GFP</i> and <i>Hprt</i> in a very small proportion of cells, and conclude that <i>Xist</i>, though not essential for the maintenance of X-inactivation, contributes to the stability of the inactive state. We further showed that <i>Xist</i> RNA, histone deacetylation and DNA methylation act synergistically to achieve the extraordinary stability of X chromosome silencing, with reactivation rates comparable to mutation rates in wild type cells (Chen et al., 1998).

**Synergism of X-inactivation mechanisms**

It has been shown that during the early stages of cellular differentiation, an <i>Xist</i> RNA mediated silencing mechanism initiates X-inactivation (Penny et al. 1996; Marahrens et al. 1997; Wutz and Jaenisch, 2000), and that <i>Xist</i> becomes dispensable for the maintenance of X-inactivation after subsequent differentiation (Brown and Willard 1994; Rack et al. 1994,
Csankovszki et al. 1999). However, we now present direct evidence that an Xist RNA mediated maintenance mechanism contributes to silencing in somatic cells. Deletion of Xist reduces the histone macroH2A1 content of Xi chromatin (Csankovszki et al., 1999), and it is possible that this change in histone composition leads to compromised efficiency of silencing. However, to directly assess the role of histone macroH2A1 in X chromosome inactivation, analysis of a targeted mutation of the gene will be necessary.

DNA methylation is another significant contributor to silencing. Demethylation by 5-azadC has been used before to reactivate genes on Xi (Mohandas et al., 1981; Graves, 1982). In this study, we also introduced a Dnmt1 mutation which proved to be about 100 times as effective as 5-azadC treatment in achieving reactivation of GFP. Demethylation combined with Xist deletion increased reactivation rates to a greater extent than either alone, indicating synergism of silencing mechanisms in keeping Xi silent. In the case of the GFP transgene we accomplished up to 30% reactivation in Xist/Dnmt1 double mutant fibroblasts. However, at least in the case of Hprt, the majority of the cells maintained silencing even after deletion of Xist and demethylation, indicating the presence of additional stabilizing mechanisms, such as late replication.

Partial compensation for reduced methylation on Xi by other silencing mechanisms occurs in ICF patients. Xi in these patients lacks methylation on CpG islands (Hansen et al., 2000) and therefore presumably histone deacetylation is also compromised. However, Xist RNA localizes normally to Xi, and together with late replication, is able to maintain X-inactivation although with reduced efficiency (Hansen et al. 2000).

Inhibition of histone deacetylation by TSA led to a small increase in the number of GFP reactivants. One possible explanation for the modest effect of histone deacetylase inhibitors is the slow rate of histone acetate turnover on the Xi of differentiated cells leading to limited enrichment in acetylated histones after TSA treatment (Keohane et al., 1998). Indeed, treatment of cells with another histone deacetylase inhibitor, Sodium butyrate, did not increase anti-acetylated histone staining on Xi (Jepesen and Turner, 1993). Furthermore, TSA only inhibits GFP silencing in demethylated cells. A similar dependence of reactivation of genes by TSA on demethylation has
been observed by others studying genes silenced in cancer (Cameron et al., 1999), implying that in these cases DNA methylation is the primary mechanism that recruits histone deacetylases to the silenced loci, possibly via binding of methyl-DNA binding proteins (Jones et al., 1998; Nan et al., 1998). However, in other cases, histone deacetylase inhibitors alone were sufficient to achieve reactivation, such as reexpression of the FMR1 gene in cells derived from fragile X syndrome patients, although synergism of histone deacetylation and DNA methylation was still observed (Chiurazzi et al., 1999).

The synergism of multiple silencing mechanisms to assure a highly stable repressed state possibly reflects the importance of dosage compensation for the proper functioning of the organism. It has been demonstrated that gene dosage imbalance in the embryo (Takagi and Abe, 1990), or in the extraembryonic tissues (Marahrens et al., 1997) causes lethality. However, partial reactivation of the chromosome appears to be tolerated in vitro and it is possible to isolate and culture cells with reactivated X-linked genes. Yet, our fibroblast clones with reactivated Xi-linked genes grew more poorly than fibroblasts of the same genotype that did not reactivate any gene. These results argue that gene dosage imbalance is detrimental to cell growth even in vitro and that clones that reactivated the entire chromosome may not be viable. Therefore, our calculations of reactivation frequencies might be an underestimate, as cells with reactivated chromosomes may die before they can be detected.

**Xist RNA and DNA methylation contribute differently to silencing of two X-linked genes**

The two genes analyzed, GFP and Hprt, are both subject to X-inactivation, but the reactivation rates are different. In the presence of Xist RNA and wild type levels of methylation and histone H4 acetylation, Hprt is almost never reactivated, while GFP reactivation can be readily observed. The difference can be at least partially attributed to the difference in assays. While reactivation of GFP can be observed almost instantly on FACS, detection of Hprt reactivants by HAT selection requires that the cells survive reactivation and maintain their proliferative capacity.
The smallest detectable HAT resistant colony contained about 30 cells, the result of about five cell divisions.

The transgenic nature of GFP might also influence the way the gene responds to silencing mechanisms. Xist RNA may not be able to exert the same level of control over a transgene as over endogenous genes, therefore deletion of Xist has a smaller effect on GFP reactivation than on Hprt reactivation. On the other hand, DNA methylation contributes significantly to the silencing of both genes. Randomly integrated transgenes are frequently methylated (Hertz et al., 1999) which may contribute to their variable expression. GFP is integrated near the centromere, and therefore might also be subject to silencing by centric heterochromatin. Nonspecific silencing of GFP by mechanisms unrelated to X inactivation (Eggan et al., 2000) may explain the rapid disappearance of GFP reactivants from the cultures. The observed influence of different mechanisms on Hprt may more faithfully represent silencing of endogenous X-inactivated genes.

**Mechanism of X chromosome silencing**

In recent years significant progress has been made in deciphering how DNA and histone modifications regulate transcription. Methylation of DNA is thought to change accessibility of chromatin via binding of methylated DNA binding proteins that in turn can recruit histone deacetylases (Jones et al., 1998; Nan et al., 1998). Acetylated amino-terminal lysines of histones, aside from affecting compactness of chromatin packaging, can bind the bromodomain module present in a wide range of chromatin remodeling proteins (Dhalluin et al., 1999; Jacobson et al., 2000).

The mechanism of Xist RNA mediated silencing is less well understood. Xist RNA is involved in preferential localization of histone macroH2A1 to Xi (Csankovszki et al., 1999). However, we do not know whether histone macroH2A1 and Xist RNA interact directly and to what extent histone macroH2A1 participates in silencing. It has been shown that Xist RNA can silence in the absence of DNA methylation (Panning and Jaenisch, 1996), or even prior to cellular differentiation (Wutz and Jaenisch, 2000). Xist RNA can also accomplish transcriptional silencing.
without the chromosome becoming late replicating or hypoacetylated (Wutz and Jaenisch, 2000). The current study provides evidence that the Xist RNA mediated silencing mechanism act synergistically with other silencing factors. Xist RNA, similarly to DNA methylation, appears to act in a localized manner, not as a master switch regulating the entire chromosome. Our observations on the effects of deletion of Xist and earlier studies on 5-azadC reactivated clones (Graves, 1982; Mohandas et al., 1981) indicate that reactivation of genes on Xi is neither coordinate nor independent, as reactivation of one gene correlates with reactivation of other linked genes only to a limited extent. Xist mutant clones with reactivated genes, in which the lack of Xist RNA mediated silencing has phenotypic consequences, provide a useful reagent for further dissecting out how Xist RNA might accomplish silencing.

MATERIALS AND METHODS

Mice and preparation of mouse embryonic fibroblasts. The Xist\textsuperscript{lox} (Csankovszki et al., 1999), the Xist\textsuperscript{a} (Marahrens et al., 1997), the GFP mice (Hadjantonakis et al., 1998) and the Hprt\textsuperscript{a} mice (Hooper et al., 1987) have been described elsewhere. To obtain mice carrying the GFP transgene and the Xist\textsuperscript{lox} allele in cis, germline recombinants were generated from double heterozygous females. Recombination frequency was 40% (n=115). Similarly, we obtained mice with the Hprt\textsuperscript{a} and Xist\textsuperscript{a} alleles in cis from mice heterozygous for both mutations in trans (27% recombination frequency, n=81). Finally, Xist\textsuperscript{a}, Hprt\textsuperscript{a}\slash Xist\textsuperscript{+}, Hprt\textsuperscript{a} females were mated to Xist\textsuperscript{lox}, Hprt\textsuperscript{+}; GFP\slash Y males to generate Xist conditional mutant fibroblasts and Xist\textsuperscript{+}, Hprt\textsuperscript{+}; GFP\slash Y males to generate controls. The Dnmt\textsuperscript{lox} (Jackson-Grusby et al., 2001) and Dnmt\textsuperscript{a} (Lei et al., 1996) mutations were bred into the colony to create Xist, Dnmt\textsuperscript{a} double conditional knockout fibroblasts.

Primary mouse embryonic fibroblasts were derived from dissociation and trypsinization of E14 embryos, cultured and when necessary immortalized with SV40 T-antigen (Jat et al., 1986). For Cre mediated recombination in fibroblasts, cells were infected with an Adenovirus vector carrying the gene for Cre recombinase (Anton and Graham, 1995). Infection was carried out in
monolayer culture in DMEM/2% fetal calf serum for two hours. The lowest possible multiplicity of infection that yielded 100% recombination without cytotoxic effects was experimentally determined. Uninfected cells were treated identically, without the addition of virus. After infection, cells were grown in DMEM/15% fetal calf serum with antibiotics. When appropriate 5-azadC (Sigma) was added to the cultures to a final concentration of 0.3 μM, and TSA (Sigma) to final concentration of 500 nM.

**Southern blotting.** To analyze the efficiency of Cre mediated Xist deletion, genomic DNA of infected cells was digested with XbaI, blotted and hybridized with probe 7, a 1.1 kb EcoRI-XbaI fragment at the beginning of exon 7. To analyze recombination at the Dmmt1 locus, genomic DNA was digested with SpeI and hybridized with the HV probe (Jackson-Grusby et al., 2001). To analyze demethylation of genomic DNA, a HpaII digest was performed and the blot was hybridized with a probe covering the gag coding region of IAP element (nucleotides 1570-1899, Accession # M17551) (Walsh et al., 1998).

**FACS analysis.** Fibroblast cultures were trypsinized to obtain single cell suspension and resuspended in complete medium. Propidium iodide was added to 1μg/mL. Viable cells were gated using scatter properties and exclusion of propidium iodide. 100,000-500,000 cells were analyzed for each bulk sample, and 10,000 cells for poorly growing HAT resistant clones. To isolate GFP positive clones, single GFP positive cells were sorted into wells of a 96-well plate containing DMEM/15% fetal calf serum. After 2 weeks, 20-30% of the wells contained fibroblast clones, of which about 30% were GFP positive or contained a high percentage of GFP positive cells.

**HAT selection and calculation of reactivation frequencies.** For selection of clones carrying a reactivated Hprt gene, the appropriate number of cells were plated and selected in 1X ESQ HAT (hypoxanthine/aminopterin/thymidine) (Stratagene) containing media for 14 days. After selection, HAT resistant clones were picked into regular media. For counting the number of HAT resistant clones, plates were fixed in methanol/acetic acid (3:1) and stained with Giemsa. For Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943), small independent fibroblast
cultures were expanded to the appropriate size, plated at a density equivalent of a 1:9 to 1:6 split in HAT containing medium, and selected, fixed and stained as above. Reactivation rates were calculated according to the P0 method of Luria and Delbrück: rate = -ln(proportion of negative cultures)/average culture size (Luria and Delbrück 1943)

Analysis of replication timing. Cells were grown in the presence of 30 μM BrdU for the last 5 hours prior to fixation. Colcemid (Sigma) was added for the last hour. Cells were fixed in methanol/acetic acid (3:1), and dropped onto slides. Slides were denatured in 70% formamide/2X SSC at 70-74°C for 2 minutes. BrdU signal was detected using a monoclonal anti-BrdU antibody (Becton Dickinson) followed by fluorescein conjugated anti-mouse antibody (Vector) in blocking buffer (1X PBS, 5% goat serum, 0.2% Tween, 0.2% fish skin gelatin). Slides were washed in 1X PBS/0.2% Tween, dehydrated and used for DNA FISH without further denaturation of the chromosomes. The Xi was identified with a directly labeled GFP/Pgk-Puro probe. The inserts of EGFP-N1 plasmid (Clontech) and pPGKpuro were isolated, pooled and labeled with Cy3- dCTP (Amersham) using random priming. Using only the GFP insert as a probe yielded identical results, but the signals were weaker. Only results obtained with the GFP/Pgk-Puro probe are shown. Hybridization of the probe and washing were performed as described (Panning and Jaenisch, 1996).

ACKNOWLEDGEMENTS

We thank Glen Paradis for help with FACS, Nicki Watson for help with microscopy, David Humpherys for the preparation of the IAP probe, Anton Wutz, Ted Rasmussen, Sandra Luiknhuis and David Akey for discussions and for critical reading of the manuscript. This work was conducted using the W.M. Keck Foundation Biological imaging facility at the Whitehead Institute. The work was supported by a grant to R.J. from the National Institute of Health/National Cancer Institute (R35-CA144339).
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**Table I.** Synergistic effect of X chromosome silencing mechanisms on the repression of GFP and Hprt.

<table>
<thead>
<tr>
<th>Xist RNA</th>
<th>histone macroH2A enrichment</th>
<th>DNA methylation</th>
<th>histone hypoacetylation</th>
<th>fold increase GFP</th>
<th>fold increase Hprt</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1x</td>
<td>1x</td>
</tr>
<tr>
<td>Δ</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>2-3x</td>
<td>100x</td>
</tr>
<tr>
<td>+</td>
<td>5-azadC</td>
<td>+</td>
<td>+</td>
<td>19x</td>
<td>12x</td>
</tr>
<tr>
<td>+</td>
<td>5-azadC</td>
<td>+</td>
<td>TSA</td>
<td>1x</td>
<td>N.D.</td>
</tr>
<tr>
<td>Δ</td>
<td>-</td>
<td>5-azadC</td>
<td>+</td>
<td>30x</td>
<td>4,800x</td>
</tr>
<tr>
<td>+</td>
<td>5-azadC</td>
<td>TSA</td>
<td>29x</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Δ</td>
<td>-</td>
<td>5-azadC</td>
<td>TSA</td>
<td>60x</td>
<td>N.D.</td>
</tr>
<tr>
<td>+</td>
<td>∆</td>
<td>+</td>
<td>∆</td>
<td>1,500x</td>
<td>N.D.</td>
</tr>
<tr>
<td>Δ</td>
<td>-</td>
<td>∆</td>
<td>+</td>
<td>2,500x</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Fold increase in the number of GFP and Hprt expressing cells following deletion of Xist and/or Dnmt1, inhibition of DNA methylation using 5-azadC, and/or inhibition of histone deacetylation using TSA. Basal level of reactivation in untreated wild type cells is designated as 1x.

*Although not tested in this study, Xi chromatin was presumed to be enriched in histone macroH2A in Xist wild type cells, and unenriched in Xist mutant cells.

+: intact; Δ: a null mutation in the gene Xist or Dnmt1; 5-azadC: cells were demethylated using 5-azadC; TSA: histone deacetylation was inhibited; N.D. not done
Table II. *Hprt* reactivation rates.

<table>
<thead>
<tr>
<th>Xist RNA</th>
<th>DNA methylation</th>
<th># independent cultures</th>
<th># positive cultures</th>
<th>average # cells/culture</th>
<th>reaction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>46</td>
<td>1</td>
<td>$3.55 \times 10^6$</td>
<td>$6.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>+</td>
<td>5-azadC</td>
<td>46</td>
<td>33</td>
<td>$3.13 \times 10^6$</td>
<td>$4.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>Δ</td>
<td>+</td>
<td>49</td>
<td>36</td>
<td>$1.32 \times 10^6$</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Δ</td>
<td>5-azadC</td>
<td>90</td>
<td>54</td>
<td>$1.77 \times 10^4$</td>
<td>$5.2 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

*Hprt* reactivation rates were determined using the P0 method of Luria and Delbrück. A number of independent cultures of *Xist* conditional mutant fibroblasts infected with Adenovirus-Cre (Δ) or left uninfected (+) were expanded to the appropriate size. When appropriate, cultures were treated with 5-azadC to inhibit DNA methylation. After HAT selection, the number of cultures with HAT resistant clones was determined and *Hprt* reactivation rates were calculated.
Figure 1. Generation of Xist conditional mutant fibroblasts with X-inactivated GFP and Hprt genes. (A) Map of the X chromosome with approximate genetic distances between genes. (B) Genotypes of Xist conditional mutant and control fibroblasts. Both cell types are phenotypically GFP negative and HAT sensitive, as the GFP transgene and the only functional Hprt allele are on the inactive X (Xi) and are inactivated (i). Conditional mutant cells carry the Xist2lox allele on Xi, while controls carry a wild type Xist allele. (C) Cre mediated deletion of Xist after Adenovirus-Cre infection. Southern blotting of XbaI digested DNA hybridized with probe 7 (pr7) indicates 100% recombination in primary (1°) and SV40 T-antigen (Tag) immortalized cells.
Figure 2. Reactivation of GFP in Xist mutant fibroblasts. (A) FACS analysis of cells. Live cells were gated and their GFP fluorescence was plotted against autofluorescence. Dots to the right of the diagonal represent cells in which GFP fluorescence is greater than autofluorescence and therefore are considered GFP positive. GFP negative (+/Y), and GFP positive (GFP/Y) populations are shown for control. Xist conditional mutant cell populations prior to Cre mediated deletion of Xist, contained a small number of GFP positive cells. After Cre mediated deletion of Xist, the number of GFP positive cells increased. (B) Number of GFP positive cells in primary conditional mutants and in controls with or without Adenovirus-Cre infection on day 7 postinfection. In Xist conditional mutants Cre mediated deletion of Xist led to a 2-fold increase in GFP positive cells, while controls remained unchanged. (C) Long term culture of SV40 T-antigen transformed conditional mutant cells. FACS analysis was performed at various timepoints following Adenovirus-Cre infection. While initially we observed an increase in the number of GFP positive cells after Cre mediated deletion of Xist, the number decreased after another week in culture to reach the level of spontaneous reactivation and remained at that level for the duration of the experiment.
Figure 3. Reactivation of Hprt in Xist mutant fibroblasts. (A) HAT selection of Xist conditional mutant and control SV40 T-antigen immortalized fibroblasts with or without Adenovirus-Cre infection. 2x10^6 cells were plated, selected in HAT media, then fixed and stained. Cells that reactivated the Xi-linked Hprt gene were able to proliferate and form colonies. No or very few colonies were observed on control plates and plates containing conditional mutants prior to Adenovirus-Cre infection. However, after Cre mediated deletion of Xist in conditional mutants, HAT resistant colonies were routinely detected. (B) The proportion of HAT resistant cells in the culture does not change significantly after culturing cells for 7 and 14 days, and 1 and 3 months following Cre mediated deletion of Xist.
Figure 4. FACS analysis of *Hprt* positive clones. The number of GFP positive cells is higher in HAT resistant clones than bulk unselected cultures. Slow growing clones reactivated GFP in a higher proportion of cells than fast growing ones.
Figure 5. Late replicating inactive X chromosomes in clones with reactivated GFP and Hprt. (A) Mapping of the GFP transgene insertion site by DNA FISH. A Cy3 labeled GFP/Pgk-Puro probe (red) was hybridized to denatured chromosomes (DAPI, blue). Enlargement of a single X chromosome is shown with the centromere staining brighter with DAPI than the rest of the chromosome. The arrow shows the site of transgene integration near the centromere. The doublet signal corresponds to sister chromatids. (B) Analysis of replication timing of the inactive chromosome. BrdU incorporation into late replicating regions of the genome was detected using a monoclonal anti-BrdU antibody and fluorescein-anti-mouse antibody (green) on DAPI stained metaphase chromosome spreads (blue). Xi (arrow) was identified using the GFP/Pgk-Puro probe (red). In all clones analyzed, the Xi with reactivated genes was late replicating. (C) An enlargement of a single late replicating Xi.
Figure 6. Synergyism of X chromosome silencing mechanism. (A) FACS analysis of GFP reactivation in Xist mutant fibroblasts with or without Cre infection treated with 5-azadC and/or TSA. TSA by itself had no effect. However, 5-azadC by itself, or followed by TSA treatment, led to significant GFP reactivation. Xist deleted cells were more sensitive to the treatment than those that did not delete Xist. (B) GFP reactivation in Xist/Dnmt1 double conditional mutant fibroblasts. Control cells (Dnmt12lox/S, Xist+/Δ) delete Dnmt1 upon Adenovirus-Cre infection, while Xist mutant cells (Dnmt12lox/S, Xist2lox/Δ) delete both Dnmt1 and Xist. Both primary (1°) and SV40 T-antigen immortalized cells (Tag) were analyzed. Deletion of Dnmt1 alone led to reactivation of GFP in up to 7% of primary and 15% of T-antigen transformed cells. Deletion of Xist also in addition to Dnmt1 increased the number of GFP positive cells about 2 fold. (C) The number of HAT resistant colonies in Xist conditional mutant treated with 5-azadC. Cre mediated deletion of Xist had a more significant effect on the number of HAT resistant cells than 5-azadC treatment. However, deletion of Xist followed by 5-azadC treatment reactivated Hprt in a much higher proportion of cells than either treatment alone. Note the use of log scale.
**Figure 7.** Demethylation of genomic DNA. (A) Demethylation of genomic DNA following 5-azaC treatment of cells. Adenovirus-Cre infected or uninfected Xist conditional mutant cells were treated with 5-azaC or were left untreated. Genomic DNA was isolated and digested with the methylation sensitive restriction enzyme HpaII. The blotted DNA was hybridized with an IAP (intracisternal A particle) probe to analyze demethylation of bulk genomic DNA. Demethylation due to 5-azaC treatment is indicated by the appearance of low molecular weight bands. (B) Adenovirus-Cre mediated recombination in Dnmt12lox/S; Xist+/Δ (control) and Dnmt12lox/S; Xist2lox/Δ (mutant) cells. Only Dnmt1 recombination in SV40 T-antigen immortalized cells is shown on Southern blots of SpeI digested DNA hybridized with the HV probe (lower blot). Nearly 100% recombination is seen in both controls and mutants. The same genomic DNA samples were also digested with HpaII and hybridized with the IAP probe (upper blot). The appearance of more low molecular weight bands and the disappearance of high molecular weight bands indicate that the genomic DNA in these samples is more extensively demethylated than in 5-azaC treated cultures.
Chapter 4.

Somatic demethylation induced Xist expression and X chromosome inactivation

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Respective contributions: Laurie Jackson-Grusby generated the fibroblasts, performed the retroviral infections for all the experiments, and generated the data for Figures 1 and 7. I performed the FISH experiments, the replication timing analysis, and generated the data presented on Figures 2 through 6 and Table 1.

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ABSTRACT

*Xist* is the gene responsible for the initiation of X chromosome inactivation. In somatic cells, the *Xist* gene on the active X chromosome is silenced by methylation. Using mouse embryonic fibroblasts containing a conditional mutation in DNA methyltransferase 1, we analyzed the effect of global genomic demethylation on *Xist* and the active X chromosome. We found that upon demethylation, the silent copy of *Xist* on the active X is reexpressed, and the RNA associates with the chromosome in cis. Tsix RNA, an antisense transcript at the *Xist* locus, is not expressed upon demethylation. The majority of *Xist* expressing chromosomes remain active. However, X-linked gene expression declines and occasionally the replication timing of an X chromosome shifts to late S phase, which is a hallmark of X inactivation. Furthermore, blocking X-inactivation in these cells by introducing an *Xist* mutation partially rescues the reduced proliferative capacity phenotype caused by demethylation. These data indicate that X chromosome inactivation can be initiated after cellular differentiation, albeit with reduced efficiency.

INTRODUCTION

Methylation of CpG dinucleotides of mammalian DNA is involved in epigenetic regulation of expression of imprinted genes (Li et al., 1993), X chromosome inactivation (Graves, 1982; Jaenisch et al., 1998; Mohandas et al., 1981) and may also be involved in tissue specific gene regulation and cellular differentiation. In the mouse, cell type specific methylation patterns are established concomitantly with differentiation by the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b (Okano et al., 1999), and thereafter maintained by Dnmt1 (Gruenbaum et al., 1982). All three enzymes are essential for mammalian
development in vivo and differentiation of embryonic stem cells in vitro (Li et al., 1992; Okano et al., 1999). Lethality of the Dnmt1 mutations is likely caused by deregulation of multiple cellular processes. The effect of Dnmt1 mutations on global methylation patterns have been previously analyzed in Dnmt1 deficient embryos and differentiating Dnmt1 mutant embryonic stem (ES) cells (Lei et al., 1996; Li et al., 1992) and in fibroblasts containing a conditional mutation in Dnmt1 (Jackson-Grusby et al., 2001). The phenotypes caused by the constitutive Dnmt1 mutation in differentiating embryonic cells include deregulation of imprinted genes (Li et al., 1993) and of Xist (Beard et al., 1995; Panning and Jaenisch, 1996) and apoptotic cell death (Li et al., 1992). Similarly, Cre mediated deletion of a conditional allele of Dnmt1 in fibroblasts leads to demethylation of genomic DNA in cultured fibroblasts, deregulation of expression of up to 10% of genes and uniform p53-mediated apoptosis (Jackson-Grusby et al., 2001). Inactivation of p53 partially rescues the mutant fibroblasts and allows about five more population doublings in culture (Jackson-Grusby et al., 2001).

Methylation is also involved in regulation of X chromosome inactivation, since both Xist, the gene responsible for initiation of X chromosome inactivation, and X-inactivated genes, are known to be regulated by DNA methylation (Beard et al., 1995; Graves, 1982; Mohandas et al., 1981; Panning and Jaenisch, 1996). X chromosome inactivation is the process that equalizes X-linked gene dosage between XX females and XY males in mammals. The inactive X chromatin assumes properties of heterochromatin in that it remains condensed in interphase (Barr, 1962), replicates late in S phase (Priest et al., 1967), and is hypoacetylated on histone H4 (Jeppesen and Turner, 1993). The inactive X is also methylated on CpG islands (Norris et al., 1991), and enriched in histone macroH2A1 (Costanzi and Pehrson, 1998). X chromosome inactivation is initiated at the time of cellular differentiation, both in vivo and during in vitro differentiation of ES cells (Monk and Harper, 1979). After X-inactivation has been established, the active and inactive states of the X chromosomes are clonally inherited.
The *Xist* gene encodes a functional, nuclear RNA that is essential for the initiation of X-inactivation (Marahrens et al., 1997; Penny et al., 1996). In undifferentiated cells, the *Xist* locus is transcribed in both sense (*Xist*) and antisense (*Tsix*) orientations to a low level from the single X of male and both X chromosomes of female cells (Lee et al., 1999). As cells differentiate, the *Xist* transcript is stabilized from the future inactive X (Panning et al., 1997; Sheardown et al., 1997), followed by cessation of transcription of *Xist* from the active X and of *Tsix* from both X chromosomes (Lee et al., 1999). In fully differentiated cells, *Xist* is expressed only from the inactive X of female cells (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). *Xist* RNA associates tightly with the inactive X, occupies the same nuclear territory and paints the entire chromosome (Clemson et al., 1996).

Methylation of CpG dinucleotides is an important regulator of X-inactivated genes and of *Xist* itself (Beard et al., 1995; Graves, 1982; Mohandas et al., 1981; Panning and Jaenisch, 1996). The promoter region of the silent *Xist* allele on the active X and CpG islands of repressed X-linked genes on the inactive X are methylated (Norris et al., 1991; Norris et al., 1994). Importantly, the methylation and expression patterns of these genes are linked to differentiation and the establishment of X-inactivation (Lock et al., 1987; McDonald et al., 1998). The importance of methylation for the silencing of *Xist* has been demonstrated by experiments in which DNA methylation was inhibited either using the pharmacological demethylating agent 5-azadC or by mutational inactivation of *Dnmt1*. Demethylation induced mouse Xist and human XIST expression has been observed in differentiating *Dnmt1* mutant mouse ES cells (Beard et al., 1995; Panning and Jaenisch, 1996), in 5-azadC treated mouse-human hybrid cells and human fibroblasts (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998). Genomic DNA of *Dnmt1* mutant ES cells is demethylated prior to differentiation, and as cells undergo differentiation, Xist RNA expression from the active X leads to inactivation of the chromosome in cis (Panning and Jaenisch, 1996). However, when demethylation occurs in fully differentiated cells,
such as in the case of 5-azadC treated hybrid cells and fibroblasts, Xist RNA coats the chromosome, yet it does not silence (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998). Similar conclusions were drawn from experiments in which Xist RNA is expressed from an inducible promoter in ES cells (Wutz and Jaenisch, 2000). If Xist expression was induced before or during the initial 48 hours of differentiation, chromosomal inactivation was initiated. However, if Xist was induced following this time period, silencing no longer took place, suggesting that in differentiated cells Xist RNA (Wutz and Jaenisch, 2000), even though it is localized correctly, is not sufficient for initiation of X-chromosome inactivation.

In order to determine whether deregulation of X chromosome gene expression contributes to the phenotypes caused by hypomethylation, we performed a detailed analysis of Xist RNA induced X chromosome inactivation in mouse embryonic fibroblasts that contain a conditional mutation in Dnmt1 (Jackson-Grusby et al., 2001). After demethylation induced by deletion of Dnmt1, Xist RNA is expressed from the active X in a significant percentage of cells. While Xist RNA was not sufficient to initiate X chromosome inactivation in most cells, we have three lines of evidence indicating that Xist expression can lead to X chromosome inactivation: First, X-linked genes Pkg-1 and Hprt were expressed at a lower than wild type frequency. Second, a few Xist expressing chromosomes became late replicating. Third, introducing an Xist null mutation (that precludes initiation of X inactivation) partially rescued the reduced proliferative capacity of Dnmt1 mutant cells. Therefore, inappropriate X inactivation contributes to the phenotypes caused by mutational inactivation of Dnmt1.

RESULTS

Xist expression in demethylated fibroblasts

Mouse embryonic fibroblasts containing a conditional Dnmt1 (Dnmt1<sup>lox/lox</sup>) mutation have been described before (Jackson-Grusby et al., 2001). Dnmt1 genotypes of fibroblasts
used in this study were Dnmt1<sup>2lox/2lox</sup> or Dnmt1<sup>1lox/5</sup>. The Dnmt1<sup>5</sup> allele is a constitutive null mutation in the gene (Lei et al., 1996). Deletion of Dnmt1 was achieved by infection of cells with a retroviral vector containing the genes for Cre recombinase and a puromycin selectable marker to allow for isolation of pure populations of infected cells. For controls, the same lines were infected with a retroviral vector containing only the puromycin selectable marker. Cre expression led to excision of exons 4 and 5 of Dnmt1 (resulting in the Dnmt1<sup>1lox</sup> allele) in virtually 100% of cells and to excessive demethylation of genomic DNA (Jackson-Grusby et al., 2001) and of the Xist promoter region (Fig. 1). Dnmt1<sup>1lox/1lox</sup> or Dnmt1<sup>1lox/5</sup> primary fibroblasts arrest after a few rounds of cell division, but p53<sup>−/−</sup> or SV40 T-antigen transformed cells continue to proliferate (Jackson-Grusby et al., 2001) and sufficient quantities of cells can be obtained. In this study, we used primary p53 wild type (referred to as “primary”), primary p53<sup>−/−</sup> (referred to as p53<sup>−/−</sup>) and SV40 T-antigen immortalized cells. As SV40 transformation and the p53 mutation lead to aneuploidy, a high percentage of cells in each line was tetraploid. Tetraploid male fibroblasts contain two active X chromosomes, and tetraploid female cells have two active and two inactive X chromosomes.

To study Xist RNA expression in demethylated fibroblasts, we used fluorescent in situ hybridization using a double stranded Xist probe combined with detection of X chromosome DNA. A significant proportion of male cells expressed Xist RNA (Fig. 2A and B). Similarly, in many female cells more than half of the X chromosomes expressed Xist, indicating the Xist locus is reactivated on the active X (Fig. 2 C and D). Xist expression patterns were similar in primary, p53<sup>−/−</sup>, and SV40 T-antigen transformed cells. The Xist signal overlapped with the X chromosome signal, covering its entire area, in a pattern indistinguishable from that seen in wild type female cells. In some cases, one X in a male nucleus expressed Xist RNA and the other did not; in some cases both X chromosomes in the same nucleus were painted with an Xist RNA signal.
For controls, we used male cells that contain a deletion allele of Xist (Xist\(^d\)) (Marahrens et al., 1997) and a probe that recognizes the 3' portion of Xist that is not deleted in these cells. Interestingly, an Xist signal was observed in many of the Xist mutant cells, indicating that the truncated allele is also expressed (Fig. 2E). In the truncated allele, promoter P1 (Johnston et al., 1998) of Xist is intact. The promoter of the neomycin selectable marker that disrupts the Xist gene is in the same orientation as that of Xist, leaving open the possibility that the truncated allele is transcribed from the promoter of the neomycin cassette (Fig. 2F). The Xist\(^d\) RNA signal is not as bright and dense as the wild type signal, nonetheless it is a useful marker for cells in the population that are sufficiently demethylated to express Xist and for the location of the X chromosome in the nucleus.

Next we determined the strand specificity of transcription from the Xist locus in demethylated cells. Xist transcription is known to be regulated by DNA methylation and the repressed promoter of the gene on the active X is methylated postimplantation (McDonald et al., 1998). Tsix, the RNA transcribed in the antisense orientation, originates from a CpG island (Lee et al., 1999) close to the differentially methylated DXPas34 locus (Prissette et al., 2001). This locus is also subject to postimplantation methylation on the active X; however no correlation has been observed between methylation of the locus and Tsix expression (Prissette et al., 2001). To determine whether demethylation leads to expression of Xist and/or Tsix, we generated strand specific probes and performed single stranded FISH experiments on demethylated primary, p53\(^−/−\) and SV40 T-antigen transformed cells. We detected a signal only with a probe that recognizes Xist RNA. Tsix signals were not seen in either Xist expressing cells or non expressers (Fig. 3). We conclude that transcription takes place only in the sense orientation (only Xist RNA is transcribed), and that demethylation of the locus is not sufficient to express Tsix RNA. Interestingly, our results indicate that while methylation is an important regulator of Xist expression, it does not appear to regulate expression of Tsix.
Expression of X-linked genes in demethylated fibroblasts

We next examined expression patterns of X-linked genes to determine whether Xist expression is sufficient to induce silencing of the X chromosome. To avoid complication due to the presence of the inactive X in female cells, we used only male primary, p53<sup>−/−</sup>, and SV40 T-antigen transformed cells for these experiments. We performed double labeling RNA FISH experiments with probes for Xist and X-linked genes P<sub>gk-1</sub> or H<sub>prt</sub> (Fig. 4). The Xist RNA signal was used as a marker for the position of the demethylated X chromosome in the nucleus. As controls, we used cells that express the mutant Xist allele, Xist<sup>R</sup>, that is known to be unable to silence the chromosome (Marahrens et al., 1997). P<sub>gk-1</sub> expression was observed both from chromosomes that do not express Xist and from ones that do. P<sub>gk-1</sub> signals frequently overlapped with both wild type and mutant Xist RNA signals. We observed similar patterns for H<sub>prt</sub> RNA. Similarly to previous data, these results indicate that Xist RNA is not sufficient to initiate silencing of the chromosome when expressed in differentiated cells (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998; Wutz and Jaenisch, 2000).

It is possible that Xist RNA can nonetheless initiate further changes in the X chromatin of differentiated cells that can result in X inactivation in some of the cells. To test this hypothesis, we determined the percentage of wild type Xist signals that overlapped with a P<sub>gk-1</sub> or H<sub>prt</sub> signal and compared them to the percentage of mutant Xist signals that overlapped with X-linked gene signals (Table 1.). As the mutant Xist RNA does not silence, the percentage of mutant Xist signals that overlapped with a P<sub>gk-1</sub> signal is equivalent to the percentage of chromosomes transcribing P<sub>gk-1</sub>. In our experiments this proportion was 50-60%, which is similar to other published studies (Panning and Jaenisch, 1996). However, the percentage of wild type Xist signals that overlapped with P<sub>gk-1</sub> was significantly smaller in all three cell types examined, indicating that some of the wild type Xist expressing chromosomes silenced the P<sub>gk-1</sub> gene. Similarly, a mutant Xist signal was more likely to overlap with H<sub>prt</sub> than the wild Xist signal (Table 1.). These result indicate
that at least two X-linked genes can be silenced on Xist expressing chromosomes of
demethylated differentiated male cells. The effect on P<sub>gk-1</sub> was more pronounced than on
H<sub>p</sub>rt, possibly due its close proximity to Xist gene.

**Late replicating X chromosomes in demethylated cells**

The inactive X chromosome replicates late in S phase and asynchronously from the active
X (Priest et al., 1967). Expression of Xist in embryonic cells undergoing differentiation
leads to long range silencing of the entire chromosome in cis and the X becomes late
replicating (Lee and Jaenisch, 1997; Panning and Jaenisch, 1996; Wutz and Jaenisch,
2000). To test whether long range silencing is possible in differentiated cells, we examined
replication timing of the X chromosomes in demethylated fibroblasts. Since we were
unable to obtain a sufficient number of primary demethylated cells for the assay, these
experiments were performed using SV40 T-antigen immortalized male cells. We labeled
late replicating regions of the genome by BrdU incorporation in late S phase. Labeled
regions of the genome were detected with an anti-BrdU antibody on metaphase
chromosome spreads (Fig. 5). As the Y chromosome is late replicating (Schmidt, 1980),
its labeling with BrdU serves as a control for the late S phase time window. In control cells,
that were infected with retroviral Puro only, we frequently saw late replicating Y
chromosomes, but the X was never labeled. However, following retroviral Cre mediated
excision of Dnmt1, an occasional late replicating X chromosome was observed, usually
together with a late replicating Y chromosome. We conclude that in a small percent of cells,
demethylation induced Xist expression leads to long range inactivation of the chromosome.

**The Xist mutation does not rescue demethylated ES cells and primary
fibroblasts, but partially rescues p53<sup>−/−</sup> and SV40 T-antigen immortalized
cells**
Demethylation is detrimental to cell growth and viability. While undifferentiated male ES cells have no growth phenotype, as the cells differentiate, they die by apoptosis (Li et al., 1992). Concomitantly with differentiation, the cells express Xist RNA and inactivate their single X chromosome (Beard et al., 1995; Panning and Jaenisch, 1996). X-inactivation was proposed to contribute to the causes of cell death (Panning and Jaenisch, 1996). Similarly, in our fibroblasts, loss of methylation leads to p53-dependent apoptosis (Jackson-Grusby et al., 2001). Demethylated male fibroblasts also express Xist, and some cells inactivate the X chromosomes. It is possible that inappropriate X-inactivation in these cells also contributes to cell death. To test this hypothesis, we compared cell lines differing only in their Xist genotype. If X-inactivation contributes to the causes of cell death, a mutation in Xist that blocks X-inactivation may partially rescue demethylated cells.

First we tested whether an Xist mutation can rescue Dnmt1 mutant ES cells (Fig. 6). ES cells with a homozygous null mutation in Dnmt1 (DnmtS/S) (Lei et al., 1996) were cocultured with ES cells containing an Xist mutation in addition to the Dnmt1 mutation (Xist<sup>+</sup>, Dnmt1<sup>S/S</sup>). Each of the lines was also cocultured with wild type cells. In the undifferentiated state, the two cell lines grew equally well, and as well as wild type cells. However, when differentiation was induced by growing cells in suspension culture to form embryoid bodies, wild type cells quickly competed out Dnmt1 mutants and Dnmt1, Xist double mutants. When the Dnmt1 mutant cells were cocultured with the Dnmt1, Xist double mutant cells in suspension, we saw no difference between the lines (Fig. 6). Therefore, in differentiating ES cells, the Xist mutation does not rescue the apoptotic cell death phenotype caused by the Dnmt1 mutation.

The effect of the Xist mutation on the growth rate of demethylated fibroblasts was also examined (Fig. 7). Xist mutant and Xist wild type Dnmt1 conditional mutant fibroblasts were mixed, and infected with retroviral Cre to see the effect of demethylation on growth rates, or with retroviral Puro vector for controls. The experiment was performed
using primary, p53⁺, and SV40 T-antigen transformed pairs. The ratio of Xist⁺/Xist⁺ alleles in the mixed population did not change in retroviral Puro vector infected (methylated) cells, indicating that the intrinsic growth rates of the different lines do not differ significantly. While the ratio of Xist⁺/Xist⁺ alleles did not change in primary cells even after Cre mediated excision of Dnmt1 and subsequent demethylation, in p53⁺ and in SV40 T-antigen immortalized cells there was a slight increase in the proportion of Xist⁺ alleles. These results indicating that blocking X chromosome inactivation in demethylated male cells results in a small proliferative advantage.

DISCUSSION

We studied the effect of Xist RNA expression in Dnmt1 mutant male fibroblasts. In these cells, demethylation led to expression of Xist RNA, but not of Tsix, from the active X chromosome. Xist RNA coated the chromosome in a pattern indistinguishable from coating of the inactive X in female cells. Xist expressing chromosomes frequently expressed X-linked genes that are normally subject to X chromosome inactivation, indicating that expression of Xist RNA in fibroblasts is not sufficient to induce X-inactivation. However in some cells Xist RNA was able to elicit further changes that ultimately resulted in initiation of X chromosome inactivation and long range silencing of the entire chromosome. Chromosomes that express both Xist and Pgg-1 or Hprt may represent a transient state that eventually will lead to inactivation. Mutation in the Xist gene partially rescues the growth phenotype caused by demethylation and inappropriate X inactivation.

Demethylation induced Xist expression and initiation of X inactivation

Demethylation induced Xist RNA expression in differentiated cells has been seen before (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998). The high percentage of Xist expressing cells in this and other studies implies that methylation plays a
major role in silencing Xist. By contrast, genes silenced on the inactive X chromosome are reactivated at a much lower frequency after demethylation. The silent copy of Xist is located on a generally active chromosome in an early replicating domain which may predispose the gene to reactivation and make it particularly sensitive to reactivation by demethylation (Gartler et al., 1999; Hansen et al., 1995; Hansen et al., 1998). Expression of Tsix was not observed in our demethylated fibroblasts, indicating that in contrast to Xist, Tsix expression is not strictly controlled by methylation. Tsix and the associated DXPas34 locus and CpG island regulate events during the initial phases of X-inactivation, such as expression of Xist prior to X chromosome inactivation, X chromosome choice, and imprinted X-inactivation (Debrand et al., 1999; Lee, 2000; Lee and Lu, 1999). No role has been assigned to either Tsix or the associated locus in somatic cells. Since methylation of the locus does not take place until after implantation (Prisette et al., 2001), it is unlikely to be involved in regulation of any of the above activities.

The process of X inactivation has traditionally been divided into initiation, spreading and maintenance phases. Initiation and spreading of silencing take place during cellular differentiation, and in differentiated cells the inactive state is clonally maintained. We induced Xist RNA expression in differentiated cells, and most Xist expressing chromosomes remained active. However, we show here that it is possible to recapitulate all the steps required for initiation of X inactivation in male fibroblasts, and initiation of silencing is not restricted to female cells undergoing differentiation. Furthermore, silencing can be initiated under conditions of severely limited methylation (Panning and Jaenisch, 1996). This observation is consistent with methylation being a late event in X inactivation (Lock et al., 1987). The fact that some Xist expressing chromosomes remained active indicate that Xist expression may not be sufficient for chromosomal inactivation in somatic cells. Alternatively, these Xist expressing active X chromosomes may represent a transient state toward inactivation. However, cells that inactivate their X chromosomes are rapidly eliminated (see Figure 7), and therefore live cells in which X-inactivation has taken place
are underrepresented in our cultures. At any time point we can observe an equilibrium between Xist expression that leads to X-inactivation and X-inactivation that leads to cell death. Indeed, our calculations of the level of Xist mediated silencing in demethylated cell (Table 1) might be an underestimate, as cells that silence the X are only viable for a limited amount of time (Fig. 7).

We also show that spreading of silencing over the entire range of the chromosome can occur in somatic cells. However, the efficiency of spreading may decrease farther away from the Xist gene. The silencing effect appears more significant on Pgk-1, the gene located in the vicinity of Xist, than on Hprt. Moreover, the proportion of chromosomes that became late replicating is smaller than the proportion of chromosomes that silenced Pgk-1 or Hprt. Decreasing efficiency of gene repression farther away from Xist in our cells is reminiscent of position dependent reduction of gene expression levels on the paternal X chromosome observed in preimplantation embryos. In preimplantation embryos, as in our cells, Pgk-1 is subject to more stringent repression than Hprt (Latham, 1996; Latham and Rambhatla, 1995).

While initiation and spreading of inactivation were recapitulated in our cells, due to the cell lethal phenotype of the Dnmt1 mutation we were unable to analyze long term maintenance of silencing in the absence of methylation. However, it is well known that methylation plays an important role in stabilizing repression of X-inactivated genes (Graves, 1982; Mohandas et al., 1981). Therefore, we would expect that in our cells silenced X-linked genes can be reactivated at a higher than wild type frequency. Indeed, overlapping Xist and Pgk-1 RNA signals may result from initial repression of Pgk-1 followed by reactivation, and not from lack of initiation of silencing. In an attempt to analyze the stability of silencing, we looked at microcolonies of cells on our slides that are presumably decedents of single cells. Cells with Xist signals that did not overlap with X-linked gene signals were frequently seen in clusters, as were cells in which the Xist signal consistently overlapped with X-linked gene signals. This observation indicates that the
select or active state of an Xist expressing chromosome might be stable at least through one or a few cell divisions. However, we also saw clusters of cells that exhibited both patterns of gene expression: those with X chromosome expressing Xist only and those with X chromosomes expressing both Xist and X-linked genes.

**Initiation of X inactivation in embryonic and somatic cells**

Xist RNA, if expressed during differentiation, inactivates the chromosome in cis (Lee and Jaenisch, 1997; Panning and Jaenisch, 1996; Wutz and Jaenisch, 2000). However, Xist RNA induced X chromosome inactivation is less efficient when it is decoupled from differentiation [our results and (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998; Wutz and Jaenisch, 2000)]. The difference in the inactivating potential of Xist RNA in embryonic versus somatic cells can stem from either a difference in the RNA and its interacting factors, as has been suggested before (Clemson et al., 1998), or a difference in the chromatin of the X chromosome prior to and following differentiation. Several lines of evidence indicate that the capacity of Xist RNA to silence the chromosome is not linked to differentiation. Xist RNA can initiate silencing in embryonic cells that remain undifferentiated (Wutz and Jaenisch, 2000). Furthermore, Xist RNA continues to silence the chromosome in differentiated cells (Csankovszki et al., 2001). On the other hand, the X chromatin undergoes extensive remodelling during differentiation including alterations in DNA methylation (Jaenisch, 1997) and histone acetylation levels (Keohane et al., 1996). To concentrate histone macroH2A1 on the chromatin of the X chromosome, Xist RNA is necessary and sufficient in differentiated cells, independent of activity status of the chromosome [(Csankovszki et al., 1999) and Rasmussen, T., Wutz, A., Pehrson, J., and Jaenisch R., submitted]. However, macroH2A1 is never concentrated on the inactive X of embryonic cells, even if Xist RNA is expressed and coats the chromosome (Rasmussen, T., Wutz, A., Pehrson, J., and Jaenisch R., submitted). We, therefore, propose that it is the state of the X chromatin in
undifferentiated versus differentiated cells that determines the ease with which Xist silences the chromosome.

Previous studies in which Xist expression was induced in somatic cells failed to detect any silencing of X-linked genes, although the assays used were not quantitative enough to detect silencing in a small percentage of cells (Clemson et al., 1998; Hansen et al., 1998; Tinker and Brown, 1998; Wutz and Jaenisch, 2000). A further difference between our study and previous data is the level of demethylation of genomic DNA. While previously cells with wild type levels of methylation or limited demethylation were analyzed, our cells are heavily demethylated. During development, genomic DNA of the developing embryo is subject to waves of demethylation, followed by de novo establishment of methylation patterns (Jaenisch, 1997). The most demethylated cells are the pluripotent undifferentiated epiblast and primordial germ cells, and methylation levels increase subsequent to implantation (Jaenisch, 1997). It is possible that the demethylated X chromatin resembles the chromatin of undifferentiated cells, and as such is more responsive to the silencing effect of Xist. Mary Lyon proposed that LINE-1 elements act as booster stations along the length of the chromosome to facilitate spreading of silencing (Lyon, 1998). It is possible that demethylated LINE-1 elements of the epiblast serve as more efficient booster stations than methylated ones in somatic cells. Therefore, spreading of silencing by Xist RNA may take place in demethylated somatic cells, but not in methylated cells.

**Inappropriate X inactivation and cell death**

Mutational inactivation of \(Dnmt1\) leads to cell death. One pathway that is deregulated in \(Dnmt1\) mutants is X chromosome inactivation. Because inactivation of the single X chromosome of male cells is lethal, one may expect that blocking the X inactivation pathway could rescue \(Dnmt1\) mutants to some extent. We observed partial rescue in \(p53^{-/-}\) and SV40 T-antigen immortalized cells, but not in primary \(p53^{-/-}\) fibroblasts and
embryonic stem cells. Differentiating Dnmt1 mutant ES cells and primary demethylated fibroblasts die by p53-dependent apoptosis (Jackson-Grusby et al., 2001; Li et al., 1992). Inactivation of p53 in fibroblasts rescues the apoptotic phenotype and allows five more population doublings (Jackson-Grusby et al., 2001). It is in this time window that blocking X-inactivation is beneficial to the cells. One interpretation of this result is that p53-dependent apoptosis and X inactivation occur at the same time, and therefore the beneficial effect of an Xist mutation does not manifest unless p53-dependent apoptosis is also blocked. Fibroblasts in which both p53-dependent apoptosis and X chromosome inactivation are prevented still arrest, indicating deregulation of additional pathways. As expression of up to 10% of genes is deregulated in these cells (Jackson-Grusby et al., 2001), the cause of arrest is a complex issue.

MATERIALS AND METHODS

Fibroblast culture, retroviral infections and methylation analysis. Generation of conditional Dnmt1 mutant fibroblasts, immortalization with SV40 T-antigen, and infection with Cre retroviral supernatant, have been described elsewhere (Jackson-Grusby et al., 2001). To generate Dnmt1<sup>lox/lox</sup>, Xist<sup>ΔY</sup> fibroblasts, Dnmt1 conditional mutant males were mated to Xist<sup>ΔY</sup> (Marahrens et al., 1997) females, and embryonic fibroblasts were isolated as before. Cre mediated recombination and demethylation of genomic DNA were documented elsewhere (Jackson-Grusby et al., 2001). For methylation analysis of the Xist locus, genomic DNA was digested with BamHI and BstUI, blotted and hybridized with a 900 bp SacII/XhoI fragment at the beginning of Xist exon 1.

Cell fixation and FISH. Cells were plated onto poly-L-lysine treated multichamber slides, permeabilized with 0.1 % Triton, fixed in 4% paraformaldehyde and stored under 70% ethanol. Hybridization, washing, and detection have been described elsewhere (Panning and Jaenisch, 1996). The Xist probe was generated from plasmid pXEXTAP (a genomic fragment from intron 1 to the end of exon 7), the Pgk-1 probe from plasmid
pCAB17 (a 17 kb genomic fragment) and the Hprt probe from pRV18.6 (an 18.6 kb genomic fragment). Probes were generated using either nick translation (GIBCO) and Cy3-dCTP (Amersham), or using random priming and biotinylated cCTP (GIBCO). Biotinylated probes were detected using fluorescein avidin (Vector). X chromosomes were identified using X paint (Oncor). For detection of X chromosomes and for combined RNA/DNA FISH, paraformaldehyde fixed slides were denatured in 70% formamide/2X SSC at 70-74°C for 8 minutes prior to hybridization of the probes.

**Strand specific FISH.** The probe that detects Xist RNA was generated form plasmid pBgl5K containing a 5 kb cDNA fragment from exons 4-7. The probe that detects Tsix RNA was generated from plasmid pSalSac containing a 5 kb Sal I/Sac II fragment 3' of the end of exon 7. Single stranded riboprobes were generated by in vitro transcription (Promega). RNA was hydrolyzed to 200-500 bp fragments by adding an equal volume of 2X hydrolyzing solution (80 mM Sodium bicarbonate, 120 mM Sodium carbonate) and incubating at 65°C for 30 minutes. Hybridization, washing and detection were the same as above, with the following modifications. The probes were hybridized at 42°C overnight. Prior to washing the slides, unhybridized RNA was digested away in 25 μg/mL RNase A in buffer B (0.5 M NaCl, 10 mM Tris pH 7.5, 0.1% Tween-20) under a coverslip for 40 minutes at 37°C.

**Replicating timing.** Analysis of replication timing was performed as described (Csankovszki et al., 2001). X chromosomes were identified using X chromosome paint probe (Oncor).

**Generating Xist, Dnmt1 double mutant ES cells.** To generate Xist, Dnmt1 double mutant ES cells, the two Dnmt1 alleles were sequentially inactivated in Xist<sup>−/−</sup> ES cells (Marahrens et al., 1997) using homologous targeting. The introduced Dnmt1<sup>−/−</sup> null mutation has been described (Lei et al., 1996). ES cells were electroporated with the pMT(S)-Puro vector, selected in 2.5 μg/mL puromycin, colonies were picked and analyzed by Southern blotting as described (Lei et al., 1996). Correctly targeted clones were expanded,
electroporated with pMT(S)-hygro, selected in 120 μg/mL hygromycin, and colonies were picked and analyzed as above. Homozygous Dnmt1 mutant clones were expanded, and used for the rescue experiments. Genomic methylation levels in these clones were found to be equivalent to Dnmt1<sup>SS</sup> ES cells (clone B) using Southern blotting.

**ES cell rescue experiments.** Wild type (J1) (Li et al., 1992), Dnmt1<sup>SS</sup> (clone B) (Lei et al., 1996) and Dnmt1<sup>SS</sup>, Xist<sup>ΔΨ</sup> ES cells were cocultured in pairs. Equal numbers of cells from each line were mixed and the mixture was either grown in ES cell medium (DMEM, 15% fetal calf serum, antibiotics, non-essential amino acids, and 1000 u/mL LIF) without feeder cells, or induced to differentiate in suspension culture on bacterial plates in differentiation media (ES cell media without LIF). Cells were harvested for DNA on days 0, 2, 4, 6, 8, 10, and 13. DNA from wild type/Dnmt1<sup>SS</sup> mixed cultures was digested with EcoRI, blotted, and hybridized with probe φ5.2 to determine the ratio of the different Dnmt1 alleles (Lei et al., 1996). DNA from wild type/Dnmt1<sup>SS</sup>, Xist<sup>ΔΨ</sup> and Dnmt1<sup>SS</sup>/Dnmt1<sup>SS</sup>, Xist<sup>ΔΨ</sup> mixed cultures was digested NcoI, blotted and hybridized with probe 4 (2 kb XbaI/BgII fragment at the end of Xist exon 7) to distinguish between the different Xist alleles.

**Fibroblasts rescue experiments.** Equal number of Dnmt2<sup>lox/lox</sup> and Dnmt2<sup>lox/lox</sup>, Xist<sup>ΔΨ</sup> cells were mixed and plated. Cells were infected with a retroviral Cre/Puro vector or retroviral Puro only vectors for control. Cells were harvested on the days indicated, the DNA was digested with NcoI, blotted, and hybridized with probe 4 as above.

**ACKNOWLEDGEMENTS**

We thank Anton Wutz and Barbara Panning for discussions, and Richard Possemato for technical help. This study was conducted using the W. M. Keck Foundation Biological imaging facility at the Whitehead Institute. The work was supported by a grant to R. J. from the National Institute of Health/National Cancer Institute (R35-CA44339).
REFERENCES


Table 1. X-linked gene expression in demethylated fibroblasts

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% cells expressing Xist RNA</th>
<th>% Xist signals overlapping with Pgk-1 signals</th>
<th>% Xist signals overlapping with Hprt signals</th>
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<td>26</td>
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<td>55</td>
<td>49</td>
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<td>26</td>
<td>53</td>
<td>44</td>
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</table>

*Dnmt1* was deleted in primary (1<sup>o</sup>), SV40 T-antigen immortalized (Tag), and p53<sup>−/−</sup> *Dnmt1* conditional mutant male cells. The percentage of cells that expressed Xist, and the percentage of Xist expressing chromosomes that also expressed Pgk-1 or Hprt was determined. In all three cell types, a mutant Xist signal was more likely to overlap with X-linked gene signals than a wild type Xist signal. To address internal variability between experiments, the Xist/Pgk-1 double labeling experiment on T-antigen immortalized cells was performed four times, and the Xist/Hprt experiment on primary cell was performed twice. While the exact percentage of Xist signals with an overlapping X-linked gene signal varied about +/- 5%, a 20-30% difference between Xist<sup>wt</sup> and Xist<sup>wt</sup> was consistently seen for Pgk-1, and a 10% difference for Hprt. (200<n<500).
Figure 1. Demethylation of the Xist promoter region.
(A) Genomic DNA isolated on the days indicated following Cre mediated deletion of Dnmt1, was digested with BamHI and BstUI, blotted and hybridized with an Xist promoter probe. The region is completely methylated in wild type male cells, and about 50% methylated in wild type female cells, corresponding to the active and inactive Xist alleles. In primary (1°) Dnmt1 conditional mutant male fibroblasts prior to Cre mediated deletion of Dnmt1 (day 0), the region is completely methylated, while 5 days following recombination, significant demethylation was observed. Similar data was obtained using p53-/- and SV40 T-antigen immortalized (Tag) Dnmt1 conditional fibroblasts.

The Xist promoter region is shown schematically on part (B). The position of the probe used in (A) and methylated CpG sites in the region are indicated.
Figure 2. Xist RNA is expressed is demethylated male fibroblasts. Analysis of Xist RNA expression in demethylated fibroblasts using simultaneous detection of Xist RNA (RNA FISH, Cy3 labeled probe) and X chromosomes (DNA FISH, biotinylated probe, detected with fluorescein avidin, green) on DAPI (blue) stained nuclei. SV40 T-antigen immortalized cells are shown; similar results were obtained using primary fibroblasts and p53-/- cells. Xist RNA is not expressed in control male (A) cells, but is expressed from active X chromosomes of demethylated male cells (B), the inactive X of control female cells (C), and both active and inactive X chromosomes of demethylated female cells (D). A less bright and less dense Xist RNA signal is also visible in male cells that contain a truncated Xist allele, XistΔ (E). The structures of the wild type and mutant Xist alleles, their promoters and the location of the Xist probe are shown on (F).
Figure 3. Strand specificity of Xist expression.
A probe that recognizes exons 4-6 of Xist RNA (Cy3 labeled, red) and a probe that recognizes a downstream portion of Tsix RNA (labeled with biotin, detected with fluorescein avidin, green) were simultaneously hybridized to cells. p53-/ Dnmt1 conditional mutant cells are shown. Identical results were obtained with primary and SV40 T-antigen transformed cells. Only Xist expression was observed both in Xist+/Y (A) and XistΔ/Y (B) demethylated male cells. To ensure that Tsix RNA can be detected with our probe, the same probe preparation was also hybridized to ES cells (C) where Tsix expression was readily observed. Location of the regions recognized by the probes is shown on (D).
Figure 4. Xist and X-linked gene expression in demethylated male cells. SV40 T-antigen immortalized cells are shown; similar results were obtained using primary fibroblasts and p53-/- cells. Xist (Cy3, red) and Pgk-1 (biotin, green) expression is shown in $Xist^+/Y$ (A) and $Xist\Delta Y$ (B) cells. Xist and Pgk-1 were frequently expressed from the same chromosome.
Figure 5. Late replicating X chromosomes in demethylated male cells. BrdU was incorporated into late replicating regions of the chromosome and detected with a monoclonal anti-BrdU antibody, followed by detection with Texas red anti-mouse antibody (red). X chromosomes were labeled with an X paint probe (green) on DAPI (blue) stained metaphase chromosome spreads. In SV40 T-antigen immortalized, Dnmt1 conditional mutant fibroblasts, an occasional metaphase spread contained late replicating X chromosomes (A). In control cells, late replicating X chromosomes were never seen (B). The Y chromosome replicates late and it was frequently labeled in both mutant and control cells. The Y can be recognized by its small size and lack of visible centromeric staining with DAPI. The number of metaphase spreads with late replicating X and Y chromosomes in shown on (C).
Figure 6. The Xist mutation does not rescue Dnmt1S/S ES cells. Pairwise combinations of wild type, Dnmt1S/S, and Dnmt1S/S; XistΔ/Y mixed cultures were either grown in ES cell medium or induced to differentiate and form embryoid bodies. DNA was isolated on the days indicated, digested, blotted and hybridized with probes that can distinguish DNA derived from the different lines. All three lines grew comparably in ES cell medium. When induced to differentiate, wild type cells quickly competed out Dnmt1S/S and Dnmt1S/S; XistΔ/Y cells. When grown together, the ratio of Dnmt1S/S/Dnmt1S/S; XistΔ/Y cells did not change in culture.
Figure 7. The Xist mutation confers a slight proliferative advantage on demethylated cells.
(A) Mixed cultures of Dnmt12lox/2lox and Dnmt12lox/2lox; XistΔ/Y cells were infected with retroviral Cre/Puro or retroviral Puro. The experiment was performed using primary, p53/- and SV40 T-antigen transformed cells. Retroviral Puro infected cells were used as controls to show that the Xist wild and Xist mutant cells grow at a comparable rate prior to Cre mediated deletion of Dnmt1. After retroviral Cre infection, the ratio of XistΔ/Y/Xist+/Y cells increased slightly in p53/- and SV40 T-antigen immortalized cells, indicating that the Xist mutant cells have a small proliferative advantage over Xist wild type cells. A similar change was not observed in primary cells.
(B) Phosphorimager quantitation of the XistΔ/Xist+ ratio. The ration increased in p53/- and T-antigen transformed cells, while it remained unchanged in primary cells.
Chapter 5

Perspectives

Although in recent years significant progress has been made in elucidating the mechanism of mammalian X chromosome inactivation, much work remains to be done. Xist RNA is the only known component of the mammalian dosage compensation machinery. Therefore the best understood aspect of X-inactivation is the role of Xist and Xist regulatory elements in different phases of X-inactivation, such as counting, X chromosome choice and establishment and maintenance of silencing. How Xist RNA achieves silencing is much less well understood. It is widely believed that the RNA interacts with other factors and forms a ribonucleoprotein complex (Clemson et al., 1996; Panning et al., 1997; Panning and Jaenisch, 1998). The next challenge in the field is determining the composition and function of this Xist RNA-RNP complex.

Components of the dosage compensation complex in C. elegans and Drosophila were initially identified in genetic screens based on the sex-specific lethality phenotype of mutations in the machinery (Cline and Meyer, 1996). Performing a similar screen in mice would be very costly and labor intensive. The human XIST gene itself was discovered fortuitously in an experiment aimed at identifying genes that escape X-inactivation (Brown et al., 1991). Moreover, it is unlikely that components of the Xist-RNP complex, other than Xist, are female specific, as expression of Xist in male cells is sufficient to induce X-inactivation (Panning and Jaenisch, 1996; Wutz and Jaenisch, 2000). It is also unlikely that
expression of these factors is restricted to cells undergoing differentiation and X-inactivation, since de novo X-inactivation can be initiated in differentiated cells (Chapter 4). However, it remains possible that Xist interacting factors are more abundant in female cells undergoing differentiation.

Attempts at isolating Xist interacting factors have not been successful. *In vivo* crosslinking and immunoprecipitation experiments (Brown and Baldry, 1996) and a yeast three-hybrid assay (Rasmussen and Jaenisch, unpublished) failed to identify interesting candidate proteins. Biochemical fractionation of female nuclear extracts or pulldown experiments using biotinylated Xist antisense oligos may still yield results. Deletion analysis of the Xist gene itself could help identify regions within the gene that are important for RNA-protein interactions and therefore Xist RNA function. With the availability of the human genome sequence and much of the mouse genome sequence as well, database searches can also be performed to look for proteins that interact with RNA, such as chromodomain containing proteins (Akhtar et al., 2000). It will be interesting to find out the identity of Xist interacting factors and to see whether they include enzymes of known biochemical function, such as histone deacetylases or proteins involved in chromosome condensation.

X-chromosome inactivation is a chromosome wide regulation of gene expression. However, not all genes seem to be affected to the same extent, since there are many genes that escape inactivation (Carrel et al., 1999; Disteche, 1995) or are subject to incomplete silencing (Sheardown et al., 1996). In order to understand the mechanism of the X-inactivation, it will be important to determine on what level the dosage compensation machinery regulates the chromosome. Individual genes, or larger chromosomal domains may define units of regulation. Analyzing expression profiles of all X-linked genes on DNA microarrays (Sudbrak et al., 2001) should help determine the level of regulation imposed on each X-inactivated gene and may bring us closer to defining the extent of coordinately regulated chromosome regions. On the cytological level, the entire inactive X
is late replicating and underacetylated on histones, but it is likely that regions that contain active genes are exempt from these modifications (Hansen et al., 1996; Jeppesen and Turner, 1993). The extent of locally active chromatin can be defined by correlating gene activity profiles with analysis of replication timing and histone acetylation levels of individual loci.

Once components of the dosage compensation machinery and the chromosomal domains regulated by the complex are known, the biochemical and molecular function of the complex and its components need to be determined. The Drosophila dosage compensation complex contains a histone acetyltransferase, and X-linked gene regulation is achieved, at least in part, by acetylation of histone H4 on the male X (Akhtar and Becker, 2000). The C. elegans dosage compensation complex is similar to the Xenopus 13S condensin complex involved in mitotic chromosome condensation and it is thought that the complex functions by inducing partial chromosome condensation in interphase (Meyer, 2000). The function of the mammalian Xist-RNP complex is unknown. I will consider three possibilities here. First, Xist and its interacting factors may be an architectural component of the inactive X chromosome. Second, Xist may be involved in defining a nuclear substructure for the inactive X. And finally, Xist may be responsible for bringing chromatin modifying activities to the X chromosome.

The first possibility is that Xist and its interacting factors are integral components of the inactive X chromosome affecting packaging of the chromatin into a condensed Barr body. However, Xist can be involved in any of several hierarchical steps of DNA compaction into chromosomes. It may alter the basic structure of the nucleosome, or its packaging into 10 nm fibers, 30 nm fibers, chromosome loops, or even higher order folding. While our knowledge of the structure of the nucleosome is fairly detailed, much less is known about large-scale chromatin folding (Belmont et al., 1999). Analyzing the architecture of chromosomes on the atomic level is inherently difficult due to their extremely large size and irregularity of structure. The evidence for Xist RNA playing a role
in altering chromosome structure include the Xist-dependence of macroH2A1 localization to the inactive X and, thus formation of macrochromatin bodies (Chapter 2). Whether the interaction between Xist and macroH2A1 is direct or indirect, remains to be discovered. Determining the function of histone macroH2A1, via mutational analysis, will be important in terms of understanding the role of Xist and macroH2A1 in the formation of the inactive X chromosome structure.

It is equally possible that Xist RNA does not interact with the chromatin directly but is instead involved in setting up a specialized nuclear compartment. The fact that Xist RNA seems to associate more strongly with the nuclear matrix than with the DNA and histones of the inactive X support this idea (Clemson et al., 1996). The study of nuclear architecture on the molecular level poses even more difficulties than the study of chromosome structure, given its even larger size. Electron and light microscopy studies of the ultrastructure of the nucleus and the nuclear matrix and scaffolds, will have to be correlated with functional studies of Xist and its interacting factors.

A third possibility is that the role of Xist is to recruit chromatin modifying activities to the inactive X. The chromatin modifying activity may be part of the Xist-RNP complex, or it may be indirectly recruited to the inactive X by Xist. Known chromatin modifications of the inactive X include DNA methylation and histone hypoacetylation (Jeppesen and Turner, 1993; Norris et al., 1991). The Xist containing complex is known to be responsible for initiating heterochromatinization (Marahrens et al., 1997; Penny et al., 1996) and later to cooperate with DNA methylation and histone hypoacetylation to repress transcription on the inactive X (Chapter 3). Methylation of inactive X-linked CpG islands and deacetylation of histone in the inactive X chromatin do not occur unless inactivation is initiated by Xist. However, the Xist-RNP complex displays a silencing activity independent of these chromatin modification (Chapter 3), and therefore its function cannot be limited to recruitment of these activities. Furthermore, methylation of CpG islands and hypoacetylation of histones do not appear until several days after the onset of Xist
expression, making it unlikely that Xist is directly responsible for recruiting these activities (Keohane et al., 1996; Lock et al., 1987). It is more likely that the role of Xist in bringing these activities to the inactive X is rather indirect. DNA methylation and histone hypoacetylation may be a passive consequence of the chromatin being silenced by another mechanism.

To determine how these activities are recruited to the inactive X, the methyltransferase responsible for CpG methylation, and the histone deacetylase enzyme responsible for deacetylating histones H2, H3 and H4, will have to be identified. It is possible that the activities responsible for these alterations are specific to the inactive X, and therefore they should be active only in female cells. Alternatively, methyltransferases, or deacetylases that also affect other genomic regions could be targeted to the inactive X. If the latter is the case, the factors involved in targeting the enzymes to the chromosome may interact at some level with Xist. Once the DNA of chromatin is methylated and the histones are deacetylated, the silent state is locked in and is stably inherited.

Xist may also be responsible for introducing other, yet unidentified, modifications in the inactive X chromatin. It is clear that acetylation is not the only histone modification of functional consequence. For example, phosphorylation of histone H3 seems to play a role during mitosis (Wei et al., 1999) and gene activation (Lo et al., 2000). Recently, another histone modification, methylation came into focus when it was found to be associated with heterochromatin (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). Furthermore, histone methylation seems to correlate with histone deacetylation (Nakayama et al., 2001). It is therefore not unlikely that histone methylation will be found on the inactive X. It has been proposed that sequential histone modifications establish a "histone code" that creates a docking surface for binding of other chromatin associated proteins, such as binding of bromodomains to acetylated histones and some chromodomains to methylated histones (Berger, 2001).
The results described in the Appendix raise the intriguing possibility that DNA elements within or near the nonexpressed active-X linked *Xist* gene are involved in regulation of the replication timing of the inactive chromosome. This observation is significant since the shift to late replication timing is an early event during X-inactivation, coincident with the onset of *Xist* expression, unlike DNA methylation, and histone hypoacetylation that appear later (Keohane et al., 1996). Perhaps *Xist* RNA is directly involved regulation of replication timing and pairing of the two homologous *Xist* alleles is necessary for proper regulation. Defining the exact DNA sequences involved and their mode of action will be an important step toward understanding the mechanism of X-inactivation.

Isolation of factors that interact with *Xist* RNA, and identifying the enzymes responsible for modification of inactive X chromatin will be an important step toward deciphering the biochemical and molecular mechanism of X-inactivation. These activities affect the hierarchical structure of the chromosome, possible at several levels. The analysis of the function of these complexes will likely involve ultrastructural studies of the chromosome and the nuclear architecture around it. New methods need to be developed before molecular machines involved in folding the chromatin fiber into interphase chromosomes can be studied on the molecular level in their native context.
REFERENCES


Appendix

A *trans*-effect of the *Xist* deletion

INTRODUCTION

*Xist* and the *Xic* have been shown to be essential for X chromosome inactivation in *cis*. *Xist* is only expressed on the inactive X, and no role has been assigned to it on the active X. Moreover, Xist RNA is only essential during the initial phases of X-inactivation, and other mechanisms can compensate for its absence in somatic cells (reviewed in Chapter 1). Therefore, deletion of *Xist* from the inactive X of mouse fibroblasts does not have an effect on the replication timing or acetylation status of the chromosome (Chapter 2). While analyzing cells with an inactive X-linked *Xist* deletion, cells with an active X-linked *Xist* deletion were used as controls. As the gene is not transcribed on the active X, we expected no phenotype in the controls. However, we found that the inactive X was no longer late replicating once *Xist* was conditionally deleted *in trans* from the active X. A deletion on the active X somehow affected the replication timing of the homologous chromosome.

RESULTS

The initial observation was made in SV40 T-antigen immortalized fibroblast clones. An *Xist^{lox+}* transformed cell line was infected with Adenovirus-Cre and clones were derived from single cells. Two types of clones were isolated: clones that deleted *Xist* from the active X and therefore continued to transcribe the gene (*Xist^{lox(Xc)}*) and clones that deleted *Xist* from the inactive X and therefore no longer transcribed it (*Xist^{lox(Xs)}*). In
$Xist^{\text{lox}X\text{y}^+}$ clones, the inactive X remained late replicating, indicating that continued $Xist$ expression is not required for late replication timing (Figure 1A and Table 1). However, in $Xist^{\text{lox}X\text{y}^+}$ clones, the inactive X no longer replicated late (Figure 1B), despite the fact that it continued to express $Xist$ RNA and continued to be enriched in histone macroH2A1 (Chapter 2).

These results were later confirmed in primary fibroblasts. $Xist^{\text{lox}X\text{y}^+}$ cells that also carried a $GFP$ transgene on the same chromosome as the $Xist^{\text{lox}}$ allele were FACS sorted. GFP positive populations carried $GFP$ and the $Xist^{\text{lox}X\text{y}^+}$ allele on the active X ($Xist^{\text{lox}X\text{y}^+}$), while GFP negative cells carried these two alleles on the inactive X ($Xist^{\text{lox}X\text{y}^+}$). Cells were infected with Adenovirus-Cre to delete $Xist$. The inactive X in $Xist^{\text{lox}X\text{y}^+}$ cells remained late replicating (Figure 1C and Table 1), while in $Xist^{\text{lox}X\text{y}^+}$ cells neither X chromosome replicated late (Figure 1D). A diagram explaining the observation is shown on Figure 2A.

<table>
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**Table 1.** The number of metaphase chromosome spreads analyzed and the number of spreads with late replicating X chromosomes in each cell type.

**DISCUSSION**

Conditional deletion of $Xist$ from the active X forced the inactive homologue to switch to early replication timing. It has been hypothesized before that homologous interaction between the X chromosomes is involved in X inactivation (Marahrens, 1999). The model postulates that the $Xist$ alleles on the chromosomes physically pair and this transvection-type interaction triggers initiation of X-inactivation. Various *trans*-sensing
effects have been seen in nonmammalian organisms (Henikoff and Comai, 1998). In mammals, physical pairing of imprinted chromosomal domains (LaSalle and Lalande, 1996) and evidence for trans-communication between homologous alleles of imprinted genes have been described (Duvillie et al., 1998). Perhaps the region deleted in the Xist$^{\text{lox}}$ allele is involved in some type of pairing interaction that influences the replication timing of the chromosome.

Interestingly, a constitutive deletion of a slightly different regions of Xist (the Xist$^{\text{d}}$ allele) on the active X (Figure 2B) does not have the same effect as the Xist$^{\text{lox}}$ allele, since in Xist$^{\text{d,lox}}$ cells where the active X carries the Xist$^{\text{d}}$ allele, the inactive X is late replicating (Chapter 2). This difference between the effect the two Xist alleles may be due to the difference in the region deleted or the difference between constitutive and conditional mutations. The way to distinguish between these two possibilities is to analyze replication timing in fibroblasts generated from mice that inherited a germline transmitted Xist$^{\text{lox}}$ allele.

It is also important to determine whether in cells with an active X-linked Xist deletion, the shift to early replication timing has an effect on the reactivation frequency of X-inactivated genes. Other features of the inactive X are intact, including Xist expression, macroH2A1 enriched chromatin, histone H4 hypoacetylation, and methylation (Chapter 2). It is therefore reasonable to expect that silencing is going to be maintained, but possibly at lower efficiency. To address this question, cells need to be generated with an inactive X-linked GFP transgene and active X-linked Hprt$^{\text{d}}$ and Xist$^{\text{2lox}}$ alleles. After Cre mediated deletion of the active X-linked Xist allele in these cells, GFP and Hprt reactivation frequencies can be determined similarly to the experiments described in Chapter 3.

By making fibroblasts that are homozygous for the Xist$^{\text{2lox}}$ allele, we can also generate inactive X chromosomes that are neither late replicating (due to deletion of the active X-linked Xist allele) nor coated with Xist RNA (due to deletion of the inactive X-linked Xist allele). By treating these cells with TSA and/or 5-azadC, one can study the interaction between late replication and other X chromosome silencing mechanisms. These
experiments are now being performed in the laboratory of Prof. York Marahrens at the University of California, Los Angeles.
REFERENCES


Figure 1. The inactive X is late replicating when Xist is deleted in cis but not when deleted in trans.
(A) In Xistlox(Xi)/+ SV40 immortalized clones, the inactive X remains late replicating as indicated by incorporation of BrdU in late S phase. (B) In Xistlox(Xa)+ transformed cells, none of the X chromosomes replicate late. Note that the cells shown in (A) and (B) are tetraploid as a result of SV40 T-antigen transformation and therefore have four Xs. (C) In primary Xistlox(Xi)/+ cells, the inactive X remains late replicating. (D) In primary Xistlox(Xa)+ cells, the inactive X no longer replicates late. BrdU incorporation was detected using a monoclonal anti-BrdU antibody and Texas red anti-mouse. X chromosomes are identified using X chromosome paint probe in (A) and (B) and using an Xist BAC probe in (C) and (D). Both probes were labeled with biotin and detected with fluorescein avidin (green).
Figure 2. Active X-linked deletion of Xist causes a shift to early replication timing of the inactive X chromosome.

(A) Xist\textsuperscript{2lox/+} fibroblasts were sorted into two groups: one with the Xist\textsuperscript{2lox} allele on the active X, and one with the Xist\textsuperscript{2lox} allele on the inactive X. When Xist\textsuperscript{2lox} was deleted from the active X, the inactive X became early replicating. When Xist\textsuperscript{2lox} was deleted from the inactive X, no change was observed in replication timing.

(B) The difference between the Xist\textsuperscript{1lox} and Xist\textsuperscript{Δ} alleles is shown. In Xist\textsuperscript{Δ}, most of exons 1-5 is deleted, but the promoter is left intact. In Xist\textsuperscript{1lox}, the promoter, a 5 kb upstream region and exons 1-3 are deleted.